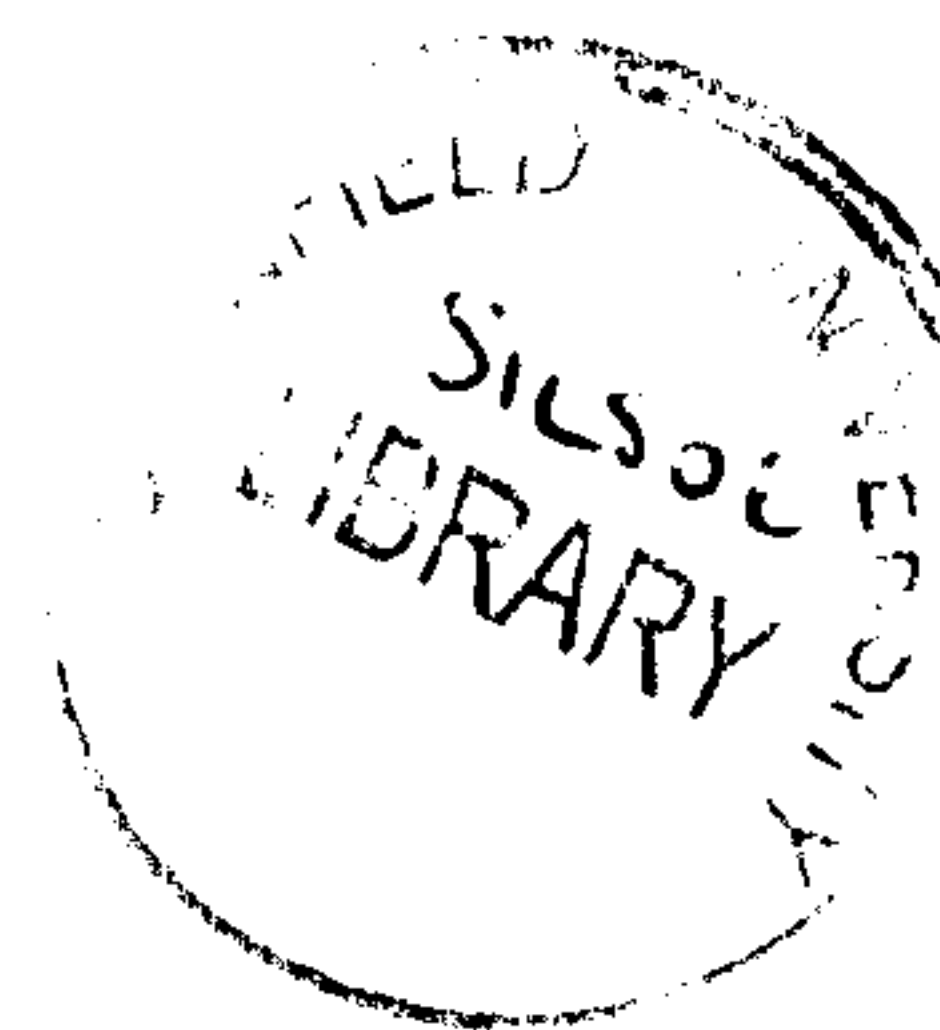


CRANFIELD UNIVERSITY
INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

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**THE DEVELOPMENT OF A SOLID PHASE FERMENTATION SYSTEM FOR
THE PRODUCTION OF PHARMACEUTICALLY USEFUL SECONDARY
METABOLITES IN FUNGI.**

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Abstract.

A *Phoma* species, producing a squalestatin (S1) was grown on agar media derived from wheat, oats, oil seed rape and malt extract over a range of water availability values corresponding to water activity (a_w) levels of: 0.998, 0.995, 0.990, 0.980 and 0.960. Growth of the organism was not significantly affected by a_w , except at the lowest value, but production of S1 was significantly enhanced at intermediate a_w in the range 0.990 – 0.980. For example, at 25°C and 0.98 a_w wheat extract produced a 10x increase in titre compared to 0.998 a_w at the same temperature. Wheat extract was shown to be the best substrate for S1 production. For example, at 25°C and 0.98 a_w , this substrate produced 2x, 5x and 8x increases in titres compared to oat, malt and oil seed rape extracts respectively.

A range of raw and processed agricultural products, including those used as extracts with the *Phoma* species, as well as maize, rice, soya, wheat flakes, bulgar wheat, couscous and “shredded wheat”, were selected as candidate materials for solid substrate fermentation (SSF). Moisture sorption isotherms were prepared for each of these so that a_w could be accurately set in experimental work. Small scale fermentations (40 cm³ wet substrate volume) were carried out with these materials and the fungi *Epicoccum nigrum*, *Sarophorum palmicola*, *Drechslera dematioidea* and *Corynespora cassiicola* over the a_w range 0.998 – 0.970. Studies with *E. nigrum* in particular produced a range of unique metabolites at low a_w , and other metabolites where titres were increased by as much as 20x compared to high a_w conditions. The optimum a_w level for metabolite production in this fungus appeared to be in the range 0.990-0.980. Ultimately, *E.nigrum* was chosen as the model fungus and bulgar wheat as the model substrate, with 3 key target metabolites being followed (metabolites 1, 2 and 3).

A series of scale-up studies (40 cm³-3 litres wet volume) were carried out utilising the model system. These studies typically produced reasonable levels of metabolites, but were subject to problematic water and heat accumulation, and bacterial contamination. These were identified as critical parameters. A system was ultimately developed around a Bioengineering AG submerged liquid fermenter, modified for use with solid

substrates, and incorporating forced aeration and mechanical agitation. This apparatus gave encouraging levels of metabolites, producing most of these rapidly and uniformly, and showed good critical parameter control.

The overall scale-up achieved in the final fermenter studies was 75x, in terms of wet substrate volume. Increased titres were achieved for all three target metabolites compared to small-scale studies with the same substrate. These increases were approx.17x for metabolite 1, approx. 3x for metabolite 3, while metabolite 2 was absent from small scale studies at the relevant a_w level.

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Abbreviations.

AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
AU	Absorption (absorbance) units
ave	Average
a_w	Water activity
BOD	Biological Oxygen Demand
bw	Bulgar wheat
cc	Couscous
cm³	Cubic centimetre
°C	Degrees celsius
ERH	Equilibrium relative humidity
FDA	Federal Drugs Administration
GMP	Good Manufacturing Practice
HPLC	High Precision Liquid Chromatography
kg, g	Kilogram, gram
l, ml	Litre, millilitre
LSD	Least Square Difference
m, mm	Metre, millimetre
min, sec	Minute, second
μ	Micro-
MAFF	Ministry of Agriculture, Fisheries and Food
MEA	Malt extract agar
med A	Medium A
NA	Nutrient agar
OEA	Oat extract agar
OSREA	Oil seed rape extract agar
OTC	Over the counter
PUF	Polyurethane foam
p	Perlite
Ret. Time	Retention time
RPM	Revolutions per minute
RO	Reverse osmosis
SLF	Submerged Liquid Fermentation
sp, spp	Species (singular and plural)
SSF	Solid Substrate Fermentation
sw	Shredded wheat
S1	Squalestatin 1
UK	United Kingdom
US	United States.
UV	Ultra Violet
WEA	Wheat extract agar
wf	Wheat flakes
WHO	World Health Organisation
w/w	Weight/weight
ψ	Water potential

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Ever since the discovery and application of penicillin in the 1940's there has been tremendous interest and debate surrounding the so-called "secondary metabolites" produced by micro-organisms. Today many of these "natural products" - as they are sometimes called - are in large scale industrial production, finding such applications as pharmaceuticals, preservatives and food additives. Typically these substances are produced by submerged liquid fermentation systems under sophisticated conditions that have been developed to yield high levels of the product.

As the search continues for new useful secondary metabolites, the large scale screening of micro-organisms for novel products is still an area of great interest, and although submerged liquid culture is probably the most widely used method, there are other alternatives which can, in some instances, be used to advantage.

One such method is solid substrate fermentation (SSF), where a solid material is used to culture the micro-organisms. This can be particularly useful in the culture of fungi, since solid materials are usually the natural substrates colonised by these organisms. The heterogeneous nature of such substrates can sometimes stimulate morphological and physiological shifts during growth which are linked to secondary metabolite production. This can lead to the production of secondary metabolites that are not observed in the homogeneous conditions that persist in liquid culture. Further, the use of SSF allows for the manipulation of environmental parameters that are difficult to alter in submerged liquid conditions. One parameter in particular is the water availability of the substrate, which has been shown to exert a significant effect on both growth and secondary metabolism in some systems.

To date however, SSF has found few industrial applications other than production of traditional fermented foods and up-grading of animal feeds. This seems to be because SSF is subject to a number of complex intrinsic difficulties concerned primarily with the heterogeneity of the systems, which lead to an inability to predict mathematically

the outcome of a fermentation. These difficulties are particularly relevant to scale-up and appear to limit exploitation of SSF on an industrial scale.

1.2. SECONDARY METABOLISM - GENERAL PRINCIPLES.

Microbial secondary metabolism is still, after some 50 years of research, an area of vigorous debate amongst workers in the field. Disagreement still persists surrounding (in particular) the origin, purposes and significance of the products of secondary metabolism, and only a few generalisations can be drawn which have gained universal acceptance. These can be summarised as follows :

1. Secondary metabolites may be defined as those products produced by micro-organisms (and other “lower” organisms) that are not directly essential for growth (Betina, 1994a). They may therefore be further defined as those metabolic products that have no known role in the “internal economy of the producer” (Williams, 1994). This contrasts with primary metabolism which may be defined as: “a summation of the interrelated enzyme catalysed reactions which are essential to growth by providing energy, synthetic intermediates and key macromolecules” (Betina, 1989).
2. They have a remarkably wide range of chemical structures (some of which are described as “unusual”), and are frequently biologically active.

3. Secondary metabolites are derived from unique, often elaborate biosynthetic pathways using intermediates of primary metabolism, e.g. amino acids, sugars, nucleosides and central pathway intermediates (Vining, 1986).
4. The enzymes of secondary metabolism are unique to these processes with substrate specificities markedly different from those of primary metabolism.
5. Production is by ordered sets of genes associated with special regulatory mechanisms that control both timing and level of gene expression.
6. These control mechanisms are remarkably well integrated with the physiology of the producer organism.

The greatest area of debate has been the role that secondary metabolites serve to the producing organisms (Aharonowitz and Cohen, 1985). Some workers have described them as “reserve products” or “detoxification products” (Vining, 1986) although other workers have pointed out the fact that many are themselves extremely toxic (Haslam, 1986). Other workers have noted that secondary metabolites are often produced when conditions no longer permit balanced growth. For example, under conditions of specific nutrient depletion, they have been described as “shunt” or “overflow” metabolites that reduce abnormal concentrations of normal cellular constituents in adverse conditions (Haslam, 1985a,b, 1986). Similarly, Ratledge (1993) has suggested that the products themselves are largely irrelevant, but the processes involved in

production allow the continuation of metabolic activity and the turnover of enzyme systems under conditions where normal growth is no longer possible. However, Williams *et al.* (1989) have extended the view that primary metabolism is itself far too finely tuned for this proposal to be acceptable. Some workers have even questioned the existence of secondary metabolism under natural conditions, suggesting that it is simply a laboratory artefact, a view discussed but not held by Maplestone (1992). However, this view seems to ignore the economic impact of secondary metabolites, such as mycotoxins, on plant crop production and storage (Betina, 1994b) which in fact is a problem great enough to necessitate legislative limits on mycotoxin levels in some food and feed substances in many countries (Moss, 1996).

The tendency for secondary metabolites to exert biological activity is well established (Bennet, 1994). Indeed some of the most potent antimicrobial drugs (antibiotics) in use today are fungal and actinomycete secondary metabolites, with penicillin and streptomycin being two well-known examples. In fact the functionality of secondary metabolites is extremely diverse, encompassing antimicrobial, anticoccidial, antiprotozoal, pesticidal, herbicidal, immunomodulatory and antitumor compounds. These represent a huge range of novel pharmaceutically relevant molecules (Vandamme, 1994) and it is true to say that the bedrock of this industry still rests firmly with these “natural products”, as they are referred to in the pharmaceutical industry.

This strong tendency towards functionality has led to a modern view of secondary metabolism, that its products confer a selective advantage to producers in the natural state (Williams, 1994). Vining (1985) has adopted this view and describes the functionality of secondary metabolites as falling into two main categories:

1. Extrinsic functions - those that impinge on growth and reproduction of other organisms in the immediate environment.
2. Intrinsic functions - those that beneficially affect growth, physiology and reproduction of the producer organism.

In fact, much has been written on the view that secondary metabolism is a process that *must* in some way benefit the producer, and the most pertinent points are summarised below :

1. Secondary metabolites are frequently produced by long, complex biosynthetic pathways (often 10-40 steps) and are extremely expensive energetically. They must therefore have been actively selected for (Williams, 1994).
2. Binding of secondary metabolites to receptor molecules is often extremely sophisticated with a complementarity approaching that of enzyme-substrate binding. This also points to evolutionary selection (Maplestone *et al.*, 1992).

3. All antibiotics so far studied are produced by genes organised into clusters often including regulatory and auto-resistance genes. Gene clustering is usually strong evidence of evolutionary selection of genes (Stone and Williams, 1992).
4. Secondary metabolism is exhibited by lower organisms lacking an immune system. It can therefore be viewed as replacing such a system in these organisms.
5. Micro-organisms living in harsh environments (where there is little competition), and nutritionally rich environments (where there is always an excess of nutrients) generally do not produce secondary metabolites (Vining, 1990; Demain, 1992).
6. The general observation that secondary metabolism is regulated to commence when balanced growth is no longer possible may reflect a specific “switching on” of various survival strategies at a time when the environment is becoming hostile and competition is at its greatest (Maplestone, 1992).

Jangen (1977) extends these ideas of competitive advantage even further by suggesting that toxic metabolites (e.g. aflatoxins) and other “objectionable” substances are elaborated onto substrates by micro-organisms to prevent their utilisation by other organisms (including man). Lillehoj (1982) has suggested the importance of ecological imbalance as a stimulus for secondary metabolite production and cites the example of high mycotoxin production in monoculture crops both pre- and post-harvest.

Secondary metabolism, being energetically expensive, is in fact highly regulated and subject to general physiological control that responds to environmental factors (Vining, 1990). It is very difficult to generalise on the factors that regulate production, but a large body of evidence suggests that it has a lower priority than growth under balanced conditions (Vining, 1990). This reflects the observation often made in submerged batch culture that secondary metabolism occurs during the “idiophase” when active growth has ceased (normally due to a limiting nutrient), and has led a number of workers to report a general regulation of secondary metabolism by growth rate itself (Hutter, 1986). Other workers report a more specific regulation exerted by the presence of readily utilisable carbon sources such as glucose, nitrogen sources such as ammonia and certain sources of phosphate. The catabolites that exert this type of control are very specific to a particular metabolite and producer. For example, although glucose is often considered a classic catabolite repressor, high levels appear to stimulate the production of aflatoxin by *Aspergillus parasiticus* (Luchese and Harrison, 1993). The mechanism of regulation seems to be repression of the enzymes of secondary metabolism therefore working at the level of DNA transcription, and is termed “catabolite repression”. In laboratory conditions, this type of regulation is lifted when the substance that exerts the effect is exhausted in the growth medium and therefore times the onset of production with changes in environmental conditions. Drew and Wallis (1983) have reported that in some systems catabolite inhibition of formed enzymes exists instead of, or perhaps as well as, catabolite repression.

It is important to stress however that the relationship between secondary metabolism and idiophase conditions does not exist for all systems and is perhaps largely a reflection of the highly unnatural conditions that prevail in laboratory culture, particularly when rich media are employed. Further, even in well understood systems where catabolite repression occurs, e.g. aflatoxin production by *Aspergillus flavus*, natural mutants can be found that produce the toxin concomitant with growth (Gendloff *et al.*, 1991). Regulation of secondary metabolism is also exerted by the same types of mechanisms that prevent over-production of primary metabolites, most commonly feedback repression and inhibition by pathway end products (Demain, 1986).

Secondary pathways that diverge from a primary pathway may also be regulated by the primary pathway end product, presumably up until the point where an imbalance in growth conditions curtails production of the end product (Betina, 1994c). In laboratory conditions production of specific secondary metabolites may also be limited by the availability of trace elements, gas balance and by the availability of light (Demain, 1986). In other systems enigmatic bioregulator molecules such as Factor A, e.g. streptomycin production by *Streptomyces griseus*, exert a powerful control on the timing of secondary metabolite production, and in others the general instability of the enzymes involved limits production markedly (Vining, 1986).

1.3. THE CONCEPT OF WATER AVAILABILITY.

1.3.1. Fundamental principles.

Many authors have written about the fundamental requirement for water in microbial growth (Scott, 1957; Cooke and Whipps, 1993). It is straightforward to describe water content of materials in terms of the percentage moisture content, based on the ratio of the dry weight to the wet weight (expressed either on a wet weight or dry weight basis). However, this tells us little about the *availability* of the water in the substrate which is accessible to micro-organisms for growth. This is principally because the water in most substrates is not all equivalent, but held in a number of different states. Broadly speaking, two main states can be identified: constitutive water, which is chemically bound to the substrate molecules, and free water, which is either absorbed or adsorbed to the substrate (Pixton, 1967). In general terms the free water becomes more weakly bound as more layers of water molecules accumulate on surfaces (i.e. as the substrate becomes wetter) and it is this weakly bound water that is normally immediately available for microbial growth. Water availability itself can be expressed in a number of ways, one of the most convenient being water activity (a_w). This is defined as the ratio of the vapour pressure of the water in a substrate to that of pure water at the same temperature and pressure (Ayerst, 1965). It is therefore linked to the equilibrium relative humidity (ERH) a sample can generate by the expression :

$$a_w = p / p_o = \text{ERH (\%)} / 100 \quad (\text{Labuza, 1974})$$

where: p = vapour pressure of water in solution or solid substrate.

p_o = vapour pressure of pure water at experimental temperature and pressure.

ERH (%) = equilibrium relative humidity at which a solution or solid substrate neither gains nor loses moisture to the atmosphere.

a_w can therefore be seen to be a measure of the ability of water to evaporate from a substrate and humidify the immediate environment, and is measured in the range 0 - 1.0 with 1.0 representing the a_w of pure water. This gives a direct measure of the proportion of water in the sample that is available for growth and metabolism, and therefore it can be seen that micro-organisms respond to a_w of a substrate rather than moisture content *per se*. Most importantly it should be noted that a_w is a function of temperature, and for a given substrate and moisture content, a_w will increase with increasing temperature. This is primarily the consequence of the general increase in thermal motion (Multon, 1988).

1.3.2. Measurement of water availability.

Practically, a_w may be measured in a number of ways, and the methods have been reviewed by several workers (e.g. Troller, 1982). Of the methods available, probably the most convenient is the electronic hygrometer (which actually measures ERH). Use of this instrument was reviewed by Kitic *et al.* (1986) who concluded that the instrument performed well, providing regular re-calibration against standard saturated salts was carried out. It is often considered difficult to work with this type of instrument at high a_w levels due to the long equilibration times required, and the risk

of damaging some types of sensors, but these problems have been overcome in the more modern instruments.

Another instrumental method for determining a_w is psychrometric measurement (reviewed by Prior *et al.*, 1976). This technique is based on measuring the rate of evaporation from a thermocouple cooled below the dew point, in a closed container humidified by the sample. This method can only be applied to samples above a certain a_w level, and is at its most efficient for very “wet” samples.

Water availability may also be expressed as water potential (ψ), which is a measure of the potential free energy in a system (relative to a hypothetical pool of pure free water of specific mass), and is measured in Pascals (Pa) (Magan and Lacey, 1988; Papendick and Mulla, 1988). The reference state of pure free water is assigned zero. Water which is “constrained” in a system, i.e. the water that is chemically or physically bound to the substrate, is therefore at a lower (negative) water potential, and any micro-organism must expend energy in terms of effecting a physiological response to raise the thermodynamic potential of the water and make it available. One of the advantages of working in terms of ψ is that the constraints in a system can be separated into their component parts, namely osmotic, matric and turgor potentials which are important parameters in some areas, particularly soil microbiology.

The numerical value of ψ may be related to a_w using the formula:

$$\psi = P + (RT/V_w) \ln a_w$$

where: P = pressure

R = gas constant

T = absolute temperature (K)

V_w = partial molal volume of water.

Effectively, ψ may be measured by both of the instrumental techniques described above.

1.3.3. Moisture sorption isotherms.

The relationship between water content and a_w in a given substrate may be usefully expressed by moisture sorption isotherms. These are sigmoidal curves produced by plotting water content against a_w at constant temperature (hence the term “isotherm”). They are highly specific to substrate type and condition, and the shape of the curve further varies depending on whether water is being progressively removed or added, due to a hysteresis effect. These plots are an important prerequisite for accurately setting a_w in a substrate by manipulating moisture content, and represent a first step in investigating microbial growth on solid substrates at varying a_w . They are normally derived via experimental means since although mathematical models have been put forward, these are complex and no universal model exists for all material types (Chen and Morey, 1989). Figure 1.1. shows typical moisture sorption isotherms for a number of agricultural materials. Figure 1.2. shows an idealised moisture sorption isotherm divided up into characteristic regions at various moisture levels.

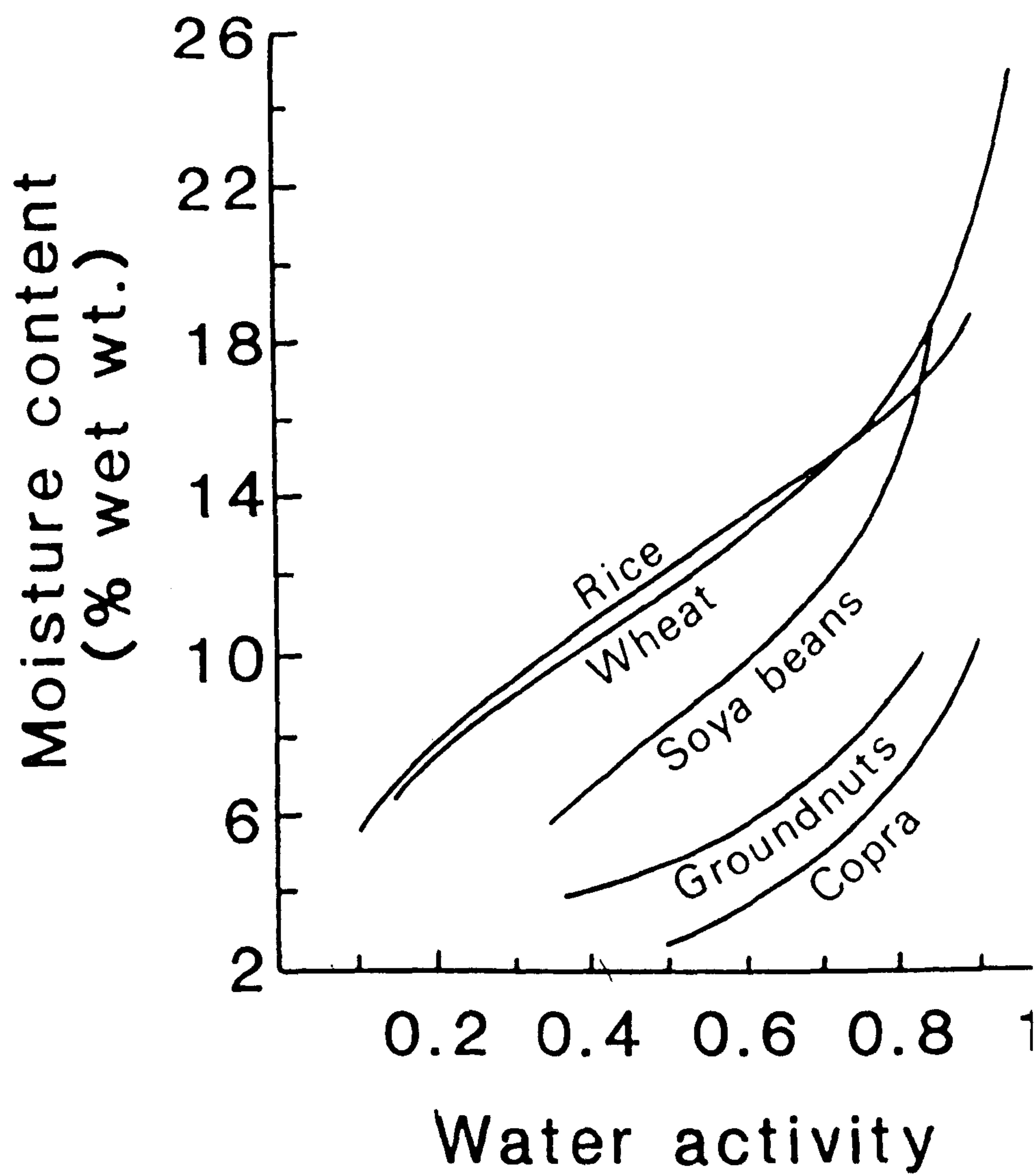


Figure 1.1. Moisture sorption isotherms for various agricultural substrates at 25°C
(Pixton, 1967).

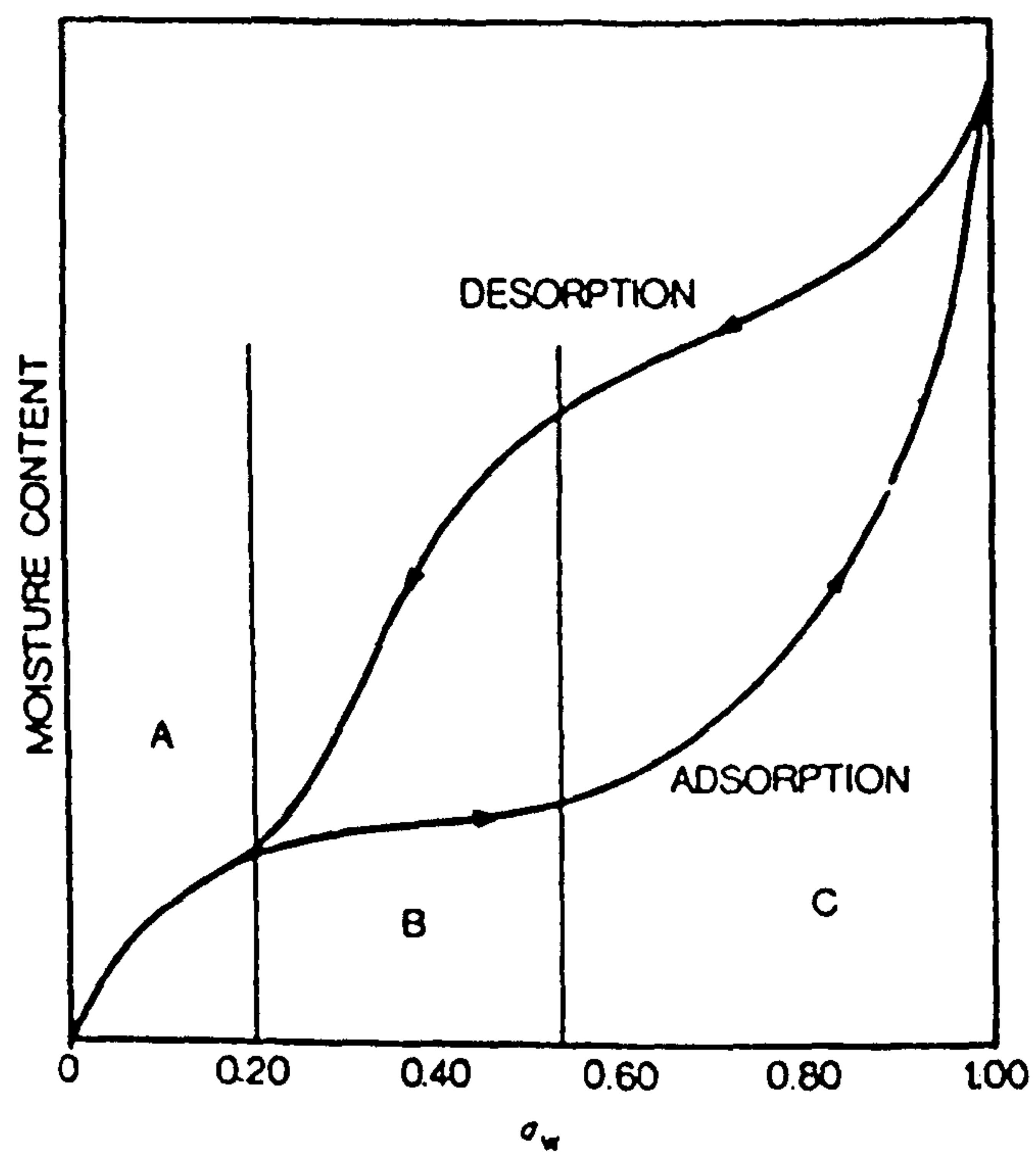


Figure 1.2. Idealised moisture sorption isotherm. (Labuza, 1974).

A - monolayer formation.

B - additional layers added to monolayer.

C - condensation in pores, capillary effects and dissolution of soluble components.

1.3.4. EFFECTS OF a_w ON FUNGAL GROWTH.

Clearly, water availability has a fundamental effect on fungal growth. Fungi generally exhibit growth at a_w levels ranging from a little below 1.0 (saturation) down to around 0.6 for extreme xerophiles (Troller and Christian, 1978). Fungi that are exposed to low a_w conditions respond in a number of ways, including rapid changes in membrane permeability leading to selective ion transport, followed by the biosynthesis of “compatible” solutes. In fungi these solutes are generally polyols such as glycerol and mannitol. In water potential terms, these changes have the effect of raising the potential energy of external water relative to internal water thus increasing its availability, i.e. the osmotic balance is altered in favour of water entering the cell (Papendick and Mulla, 1985). The effect of water availability on fungal behaviour has been well investigated, often in conjunction with other parameters such as nutrients, temperature, O_2 , CO_2 and pH. In general, sub-optimal a_w conditions lead to slower growth, slower spore germination and increased lag times. These effects have recently been reviewed by Magan (1997). Magan and Lacey (1984, 1988) studied extensively the interactions between a_w and temperature, pH, substrate type and O_2/CO_2 balance in a series of studies on grain fungi. In general they found that the lowest a_w levels for growth and germination were tolerated at optimal growth temperatures and similarly the widest temperature range for growth and germination occurred at the optimal a_w level. The separate application of temperature, pH and gas balance stresses interacted with lowered a_w to markedly inhibit growth and germination.

Wilson and Griffin (1974) looked at the effect of lowering a_w on four soil fungi. They reported four important effects:

1. Increased lag time before growth commenced.
2. Decreased respiration rate.
3. Decreased growth rate.
4. Increased O_2 uptake required for unit growth.

They concluded that at low a_w , metabolic activity was diverted away from growth into the energetically expensive biosynthesis of compatible solutes necessary to make external water available.

Cahagnier *et al.* (1993) investigated growth and conidiation at lowered a_w in two storage moulds growing on maize and paddy rice. They found that while growth was inhibited at low a_w , conidiation occurred more readily. Conidiation in these fungi may be associated with water availability “stress”. This is an important observation since conidiation is a differentiation response often linked to other secondary processes such as secondary metabolite production, and therefore provides one example of a link between stress and secondary responses.

1.4. SECONDARY METABOLISM AND ENVIRONMENTAL FACTORS.

Effects of interactions between a_w and other environmental factors on fungal secondary metabolism have been reported widely. However, little information is available on secondary metabolites of pharmaceutical interest, most knowledge being

in the area of mycotoxin production. For example, Marin *et al.* (1995) studied growth and fumonisin production by two *Fusarium* species on maize over a range of temperatures and a_w levels. They found that although there were some interspecific differences, fumonisin production occurred over a range of intermediate moisture contents (a_w levels in the range 0.97-0.93) corresponding to poorly dried maize. Temperature was found to influence mycotoxin production less than water availability. Studies by Cahagnier *et al.* (1994) on the same organism reported a straightforward decrease in fumonisin production as a_w decreased. In particular, they reported a 300 fold decrease in fumonisin production when a_w was decreased by 10 per cent (a_w 1.0 - 0.9).

Magan and Lacey (1985) studied growth and mycotoxin production by *Alternaria alternata* on wheat agar and wheat grain over a range of a_w and temperature regimes. They found that both a_w and temperature exerted a strong effect on mycotoxin production and growth separately, and had a dramatic effect on mycotoxin production when manipulated in combination. They suggested that more water was required for mycotoxin production than was required for growth alone. Environmental stress has also been demonstrated to modify production of some volatile metabolites. A study by Gervais *et al.* (1988) followed aroma (2-heptanone) production by *Trichoderma viride* on yeast - starch agar medium over a range of a_w levels. They demonstrated that production varied markedly over the a_w range 0.98 - 0.96, with maximal production at 0.98, and declining with drier conditions.

Niles *et al.* (1985) grew eight strains of *Aspergillus flavus* on wheat and barley grain and followed growth and aflatoxin production over a range of temperatures (16 - 42.5°C) and a_w levels (0.8 - 0.975). They reported that at the optimal temperature for aflatoxin production (35°C), the optimal a_w was 0.95 and above, but at sub-optimal temperatures the optimal a_w was raised to 0.975. The highest a_w level used did not inhibit mycotoxin production, but it should be noted that the highest a_w level used here (0.975) may be considered to be relatively low. A number of other groups have worked with aflatoxin production by *Aspergillus* spp. and all report a general trend for metabolite production to be severely limited by low a_w levels with maximal production at the highest levels (approx 0.98 a_w). (Northolt *et al.*, 1977, Faraj *et al.*, 1991, Asvedo *et al.*, 1993, Adebajo *et al.*, 1994). Similar trends have been reported for trichothecene production on cattle feed by various fungi (Laxma Reddy and Reddy, 1992) and for penicillic acid production by *Penicillium* and *Aspergillus* spp. growing on agar media (Northolt *et al.*, 1979).

All the preceding studies describe a fairly clear decrease in metabolite production with decreasing a_w . However, Northolt *et al.* (1979) demonstrated the ability of *Aspergillus* and *Penicillium* spp. to produce ochratoxin A at low a_w levels (0.90 - 0.83). Further, Patterson and Damoglou (1986) working with various toxigenic fungi growing on a "bread analogue" reported the accumulation of citrinin, ochratoxin A and sterigmatocystin at a_w levels they described as low (approx 0.85 at 25 °C). In the same study they described patulin requiring a minimum a_w of 0.95 for production. This observation has previously been reported by Northolt *et al.* (1978). Similar

results were reported by Roland and Beuchat (1984) with growth of *Byssochlamys nivea* in apple juice.

Etcheverry *et al.* (1994), in studies of *A. alternata* on sunflower seeds at a_w levels of 0.80, 0.87, 0.90 and 0.97 reported optimal tenuazonic acid production at 0.90 a_w and 25°C. Baxter (1997) working with a *Phoma sp.* and using agar media and cracked maize as growth substrates found that optimal production of zaragozic acid occurred at 0.995 a_w compared to 0.998 a_w , which was optimal for growth at 25 °C. In fact zaragozic acid production was decreased by as much as 10 fold at 0.998 when compared to 0.995 a_w . Maximum zaragozic acid production was observed over the range 0.995 - 0.99 a_w . Baxter (1997) also found that the ratios of different types of zaragozic acids produced by the organism were affected by altering a_w . This study also showed that the minimum a_w tolerated at the optimum growth temperature was lower than the minimum a_w tolerated at sub-optimal growth temperatures. This has previously been reported by Magan and Lacey (1984a) working with field and storage fungi of cereal grains.

A number of other environmental factors can also influence secondary metabolism. These tend to be specific to organism and metabolite, so few generalisations can usually be made. In the case of aflatoxin production by *A. flavus*, Ellis *et al.* (1994) indicated that initial O₂ level significantly influenced production of the toxin, with the maximum at 10% O₂. In particular, their work demonstrated the combined effect of a_w , temperature and O₂ level on metabolite production. Scheidegger *et al.* (1988),

working with the production of Beta-lactam antibiotics by *Cephalosporium acremonium*, had previously reported a less straightforward effect of O₂ concentration. They showed that O₂ starvation after the onset of cephalosporin-C production led to increased yields of penicillin-N whilst reducing the yield of cephalosporin by 30%. This suggests the perturbation of a common pathway by O₂ starvation. Paster and Lister (1985) working with patulin production by *Penicillium patulum*, reported reduced patulin production at both 5% and 60% O₂ concentrations compared with production at 10 - 40% O₂. However, they showed that patulin production at extreme O₂ levels recovered when 1% CO₂ was supplied to the cultures. This suggests that CO₂ was required for patulin production under conditions of high and low O₂ "stress".

Light may also influence the production of some secondary metabolites, but here the situation becomes even less clear. For example, Moss and Frank (1985), have summarised the reported effects of light on aflatoxin production, which in some instances seems to have no effect, and in others appears to be inhibitory. They also discussed the effects of interactions between micro-organisms in the production of secondary metabolites. In general (and perhaps surprisingly) the effects appeared to be inhibitory, but again some conflicting results were reported. Most interestingly they reported a study where rubratoxin (a toxic metabolite of *Penicillium purpurogenum*) significantly enhanced the production of aflatoxin by *Aspergillus parasiticus* growing on maize. It should be noted that in these particular studies, a_w of media was not modified or controlled.

1.5. SOLID SUBSTRATE FERMENTATION

1.5.1. General introduction

A critical evaluation of historical events, present day knowledge of fermentation and an appreciation of the importance of the optimum water content indicate that the decisions taken in 1940 to ignore solid substrate fermentation systems in the Western Countries were probably not appropriate (Lonsane et al., 1992).

The fixation of the Western World for deep tank pure culture (liquid) fermentation has been partly responsible for the stagnation of the growth in the fermentation industry (Hesseltine, 1972).

These two comments reflect the contrast between the technological sophistication of modern submerged liquid fermentation (SLF) systems compared to that of solid substrate fermentation (SSF), which is an area that appears to be still in it's infancy. This is perhaps surprising since SSF has a history going back many centuries in the production of certain traditional (mainly Eastern) fermented foods (Kim *et al.*, 1985). The Chinese probably started making *chiang*, a forerunner of miso, around 2.5 thousand years ago, and this was brought to Japan by Buddhist priests in the 7th Century (Hesseltine, 1977). However, in the last decade SSF has gained new attention in specific areas including enzyme production, animal feed production, solid waste

management and as a potential tool for the production of a number of chemical substances.

SSF may be defined simply as the culturing of micro-organisms on a moist solid substrate, in the absence of free-flowing water (Durand *et al.*, 1988). It is distinct from culture techniques involving agars (although these are often referred to as “solid” substrates) since agar is a *solidified* medium, and behaves as a liquid in a number of important ways.

The nature of SSF is fundamentally different from that of SLF and defies simple characterisation, due in part to the inherent complexity of microbial growth on a (normally) heterogeneous solid material. In fact, a diverse range of materials have been used in SSF (Table 1.1). Most may be characterised as insoluble lignocellulose or starchy polymers represented by agricultural products, by-products or wastes. Mineral or synthetic materials such as perlite, clays or polyurethane foams, i.e. inert supports to which nutrient substances are added, have also found limited application, (Kerem & Hadar, 1993; Durand *et al.*, 1996).

Solid substrates closely resemble the natural environment of the fungi, which have a filamentous growth habit, and generally are the organisms most suited to SSF culture techniques (bacteria being essentially aquatic) (Kim *et al.*, 1985). The closeness to natural conditions achieved in SSF systems may be significant since a number of workers, notably Hesseltine (1986), have suggested that secondary metabolite

Table 1.1. Examples of applications of SSF (Smits *et al.*, 1998).

Product(s)	Substrate	Fermenter type	Micro-organism
Animal feed	Sugar beet pulp	Packed bed, pilot scale	<i>Trichoderma viride</i>
	Coffee pulp	Packed bed	<i>Aspergillus niger</i>
	Orange peel	Bags	(mixed)
	Sago starch	Packed bed	<i>Rhizopus oligosporus</i>
Protein	Banana wastes	Stirred tank, pilot scale	<i>Aspergillus niger</i>
	Fodder beets	“mixed”, pilot scale	<i>Saccharomyces cerevisiae</i>
	Citrus peel	Packed bed	<i>Aspergillus niger</i>
	Sawdust	Tray/rotary drum	<i>Chaetomium cellulolyticum</i>
Tempeh	Soya beans	Tray/rotary drum	<i>Rhizopus oligosporus</i>
Koji	Rice/soya beans	Tray, industrial scale	<i>Aspergillus oryzae</i> etc
Cellulase	Leached beet pulp	Packed bed	<i>Trichoderma aeroviride</i>
	Wheat bran	Tray	<i>Trichoderma reesei</i>
Pectinesterase	Apple pomace	Rotating drum	<i>Aspergillus niger</i>
Hydrolase	Orange peel	Rotary drum	<i>Aspergillus niger</i>
Protease	Wheat bran	Tray	<i>Aspergillus flavus</i>
	Synthetic medium	“inert carrier” repeated batch	<i>Aspergillus oryzae</i>
Alkaloids	Bagasse	Packed bed	<i>Claviceps purpurea</i>
B-glucosidase	Sugar beet pulp	Column	<i>Aspergillus phoenicis</i>

Table 1.1. cont.

Product(s)	Substrate	Fermenter type	Micro-organism
Citric acid	Beet pulp	Tray, non-sterile	<i>Aspergillus niger</i>
Ethanol	Maize	packed bed, pilot scale	<i>Saccharomyces sake</i>
Gibberellic acid	Cassava flour	Column	
	Sugar cane bagasse	Column	<i>Gibberella fujikuroi</i>
	Synthetic medium	Column	
Lactic acid	Bagasse	Packed bed	<i>Rhizopus oryzae</i>
Ochratoxin A	Wheat	rotary drum	<i>Aspergillus ochraceus</i>
Aflatoxin	Wheat	Rotating drum	<i>Aspergillus parasiticus</i>
	Rice		
Iturin	Okara	Flasks, lab/ pilot scale	<i>Bacillus subtilis</i>
Oxytetracycline	Potato residue	Packed bed	<i>Streptomyces rimosus</i>
Penicillin	Bagasse	Packed bed	<i>Penicillium</i>
			<i>chrysogenum</i>
Bioinsecticide	Enriched clay	Packed bed	<i>Beauveria bassiana</i>
		commercial scale	

expression is best achieved where conditions are created that nearest match those that the organism finds in nature. Most importantly, the heterogeneity of most solid substrates presents the fungi with an array of discrete micro-environments and nutrient gradients which can in some cases encourage the differentiation of the fungal mycelium, the development of secondary structures and expression of secondary metabolites (Penn, 1994). In higher fungi (basidiomycetes and ascomycetes) expression of all stages in the life cycle may be possible with the potential for the production of specific, and perhaps unique metabolites (Penn, 1994). In fact, for many higher fungi SSF represents the only method available for laboratory culture, and therefore provides unique opportunities for obtaining potentially useful metabolites.

1.5.2. Critical parameters in SSF

The major parameters that influence microbial growth in SSF can be listed as follows:

1. Chemical nature of the substrate.

This is concerned with a number of parameters including the nutrient balance and the degree of polymerisation and crystallisation of the substrate. For example, cellulose, which in a number of forms probably represents the most abundant polymeric substance utilised in SSF systems, can differ markedly in the extent of crystallisation of its structure. In general, the more crystalline the structure is, the more resistant it is to degradation. Further, the ability of the organism to express appropriate enzymes to deal with insoluble polymeric material is clearly of great importance. The utilisation of insoluble polymeric material commonly requires processing by extracellular

enzymes before it can enter the cell (Knapp & Howell, 1980). Other chemical considerations are surface electrochemical properties and hydrophobicity, which will influence the way that the micro-organisms are able to interact with the surface (e.g. the ability to attach to the surface) (Knapp & Howell, 1980). Utilisation of the substrate is likely to be most efficient when the organisms are intimately associated with it.

2. Physical nature of the substrate.

This is concerned primarily with the accessible area available for microbial growth since the rate of hydrolysis is related to available surface rather than the total mass. Available surface area itself depends on a number of parameters relating to the physical condition of the substrate. The first important parameter is the particle size. Knapp *et al.* (1980) grew a *Thermoactinomyces* sp. on three particle sizes of avicel (a regenerated cellulose with spherical particles) and reported a slight increase in growth rate, with a more significant increase in total growth and cellulose utilisation, with the smallest particles used. Pandey (1992) found that enzyme productivity in a system using cassava starch particles was optimum for a particle size range of 180 μm - 1.4 mm (micro-organism unspecified). This particle size range was considered to optimise available surface area.

Hesseltine (1972), working on aflatoxin production in grain substrates reported rice kernels or pearled wheat to be an "ideal" size. He found that corn and soya beans were best broken into approx. 5 mm pieces. Larger particles are generally considered to limit fungal growth by effectively lowering surface to volume ratio and therefore

limiting the available contact area (Pandey, 1992). They may also introduce a limitation in terms of the rate of diffusion of nutrients to the surface. However, Barrios-Gonzalez *et al.* (1993) reported optimum giberellic acid production with a wheat bran particle size of 3 - 4 mm, with significantly less produced with smaller particle sizes.

The physical state of the particle surface is also an important consideration. Damaged, broken surfaces often seem to be attacked preferentially by micro-organisms (for example some rumen bacteria appear to attach preferentially to cut ends of ingested grass) and comparatively few micro-organisms seem able to attach to undamaged surfaces (Knapp & Howell, 1980).

3. *Aeration.*

In theory, adequate aeration should be easier to achieve in SSF processes than in aerobic SLF processes which are often oxygen limited due to the low solubility of oxygen in water. In SSF oxygen uptake is probably directly from the gas phase (in void spaces) and also from dissolved oxygen in water films. In practise however, aeration is as critical in SSF processes as it is in aerobic SLF processes. Aeration characteristics in static systems (i.e. where the substrate particles remain stationary) are mainly governed by substrate particle size and void space, bed thickness and packing density. These parameters limit the scale and efficiency of such systems, and most modern processes rely on some form of active aeration such as air injection and /or agitation (Section 1.5.3.).

Aeration can be a critical factor even in laboratory scale processes: Hesseltine (1972) has given a fascinating account of how he became involved in the problem of aflatoxin poisoning in the late sixties and was faced with the task of producing large quantities of the toxin for experimental work. He eventually found that “quite phenomenal” yields of aflatoxin (typically $> 1\text{ g kg}^{-1}$ substrate) were obtained by culturing the producer organism (*Aspergillus parasiticus*) on rice or pearled wheat in flasks on an orbital shaker at 188 RPM. He attributed the success of this process at least in part to the ideal aeration efficiency of the system. It must be stressed however, that his process, although also successful for production of ochratoxin A by *Aspergillus ochraceus* on pearled wheat, did not produce enhanced levels of secondary metabolites in all cases. For example, zearelanone production by *Fusarium moniliforme* was not enhanced. This probably reflects the inability of some fungal species to tolerate the shear damage experienced by the mycelium under conditions of continuous agitation (Barrios-Gonzalez, 1993). Intermittent agitation may be better for some systems, and has been shown to be just as effective, in terms of gas transfer efficiency, as continuous agitation in some cases (Lonsane *et al.*, 1992).

4. Water.

Although SSF is typically practised in the absence of “free flowing water” it is important to remember that ultimately all microbial growth depends on the presence of water, either freely available or acquired by active physiological adaptations e.g. by the xerotolerant species. Bacteria are essentially aquatic organisms so it is the fungi that are optimally suited to SSF conditions. Indeed, the important saprophytic role of fungi in nature can be viewed as a continuously occurring global SSF process. In SSF,

the absence of free water is probably more usefully described as an absence of excess water, and in most systems the solid substrate will be in a moist state with water present as a film on the particles. This water will be freely available for microbial growth, so that a clearly defined water phase will normally be present, and the typical a_w of the substrate, at least at the start of the fermentation, will be close to, or at saturation (a_w 0.995-1.00). For most phycomycetes, ascomycetes and basidiomycetes the optimum a_w for growth will fall within this range (Pandey, 1992).

For most SSF processes an optimum water content will exist for growth and/or metabolite production. In the more traditional processes this level will have been arrived at by “rule of thumb” or “trial and error” without a clear understanding of the water relations of the fungi concerned. However, with the more technologically sophisticated modern processes, a_w itself is closely monitored and regulated. The effects of allowing the water content to drop below the optimum level will have an obvious effect on growth or metabolite production, but increases in water content above the optimum can also have adverse effects. Most importantly, the substrate pore spaces may fill with water (“water logging”) effectively excluding O_2 (Moo-Young *et al.*, 1983). Also, mechanical changes such as softening and caking may occur in the substrate and there may be an increased risk of bacterial contamination, particularly in systems that are not run strictly aseptically. Moisture level can therefore be critical in many SSF processes. In particular, Pandey (1992) reported on an SSF system where a significantly lower yield of gibberellic acid and certain enzymes was obtained when the water content was above a certain limit. Further, in his account of aflatoxin

production by SSF, Hesseltine (1972) described a critical level of water to be added to rice or wheat of 25 ml : 50g to allow each grain to remain separate and move independently during shaking. This idea was itself based on the acknowledged “secret” of successful koji fermentation which apparently depends on “ making sure each rice grain is uniformly and thoroughly moistened so that it feels moist but not wet at inoculation”. These effects have been attributed to the more efficient exchange of gasses permitted by lower water levels. It is also possible however that the drier conditions can, in some cases, introduce a stress effect which may stimulate the micro-organisms to produce higher levels of secondary metabolites. For pharmaceutical screening programs, very few studies have examined the impact of a_w and it’s interactions with other environmental parameters.

1.5.3. Scale-up

In SSF, just as in SLF, scale-up is the crucial link in transferring a laboratory scale process to commercial production scale. The size of a commercial process will depend on the nature of the intended product and may range from tens of kilograms of substrate for some secondary metabolite products to thousands of tonnes of substrate for composting and biomass conversion processes (Lonsane, 1992). Scale-up itself often presents major difficulties since changes from laboratory scale operations, which may deal in gram quantities, leads to very different aeration, heat dissipation, settling and compression characteristics. Unlike SLF, there are few, if any criteria for rational SSF reaction scale-up. The approach is largely empirical, often, for example doing little more than maintaining geometrical similarity (i.e. vessel shape) as the scale increases (Durand *et al.*, 1996). Indeed, it is generally accepted that the design of a

commercial scale process cannot be accomplished solely by theoretical means, and that each potential process must be approached from first principles (Lonsane *et al.*, 1992). The major difficulties in scale-up may be listed as follows:

1. Strain variation in biomass.

The increased number of generations required to develop the microbial biomass on a large scale often generates a high degree of genetic drift with a significant proportion of non-productive strains. The extent of the problem will vary depending on the stability of the organism involved but is described by Lonsane (1992) as “ a major cause for the abandonment of a process on a production scale”. This problem also applies to inoculum development where a number of stages may be involved to generate sufficient material. This problem is also encountered in SLF processes, and it is unclear whether the SSF processes are affected to a greater extent.

2. Medium sterilisation.

In general it seems that the efficient sterilisation of solid, heterogeneous material on a large or even intermediate scale can often present problems (Dr. J. Penn, TerraGen Discovery Ltd., pers comm.). Process times may need to be increased to several hours to ensure complete sterilisation. This may lead to physical and chemical alterations in the medium and thermal degradation of certain nutrients (Lonsane, 1992). Prolonged sterilisation times will also significantly increase process costs.

3. Air supply.

At laboratory scale, using perhaps gram quantities of substrate, diffusion alone may be sufficient to adequately aerate the substrate mass. On a commercial scale, diffusion

will be insufficient to allow efficient gas transfer due partly to the physical size of the mass, and the likely compression of the pore spaces due to the increased weight. Therefore, some form of forced aeration may be necessary. This is true even of the most traditional commercial scale processes such as Koji production where air is processed and pumped into the Koji “rooms” (Smits *et al.*, 1998).

In the more sophisticated packed bed or column reactors air is sometimes forced at pressure through the substrate, usually from the base of the bed (Durand *et al.*, 1988). This clearly becomes more difficult as the amount of substrate increases, and it is often necessary to route the air flow carefully using distribution systems to prevent channelling. Channelling occurs when the air follows a preferred route through the substrate, leaving some areas unaerated. Aeration may also be achieved by agitation of the substrate, either continuously or intermittently. This has the effect of mechanically re-supplying the void space with O₂ and releasing CO₂ at the same time. A number of designs of SSF bioreactors allow for agitation of the substrate using paddles or rakes (e.g. stirred horizontal bioreactors) and others mix the contents by rotational movement of the whole substrate mass (e.g. rotating drum bioreactors). In practice, many commercial processes combine forced aeration with agitation, either continuous or intermittent (agitation is discussed further in section 4.).

4. Heat build-up.

The heterogeneous nature of SSF processes often leads to quite spectacular metabolic heat build-up within the substrate bulk (Ghildyal *et al.*, 1993). The effect is well

known in SSF processes such as composting and ensilage and can quickly lead to interior temperatures in excess of 50°C (Smits, 1998). Lonsane *et al.* (1992) have reported temperatures of 60-70°C commonly in the centre of compost heaps representing a temperature increment of +30°C compared to the exterior.

Clearly such metabolic heat development will be detrimental to most SSF processes. However, at the laboratory scale, heat build-up may not be a significant issue, particularly where thin beds of material are involved and passive heat transfer to the exterior may be adequate to maintain a stable temperature. However, for fermenter based processes, as the vessel size is increased, the working volume increases with the *cube* of the linear dimension, while the available surface area increases with the *square* of the linear dimension (Lonsane *et al.*, 1992). It can be seen therefore that as the scale increases, the efficiency of heat dissipation from surfaces must proportionately decrease, and on a commercial or even pilot scale some form of active heat removal process is usually essential. At the pilot scale efficient heat transfer may be accomplished by placing the reactor in a water bath or some other form of heat exchanger, e.g. by routing re-circulating cold water through a baffle system (Roussos *et al.*, 1993).

On a larger scale these methods become less practical, and aeration may be used as a method of heat removal. In this method a passage of air through the substrate effectively promotes evaporative cooling and, under certain conditions has been reported to be capable of removing up to 80% of metabolic heat generated (Lonsane,

1992). In theory, evaporative cooling can be made even more effective by combination with agitation (Smits *et al.*, 1998) where agitation disperses the heated material uniformly (i.e. removes “hot spots”) and promotes cooling efficiency. Aeration on a large scale as a means of heat removal will only be effective if the substrate is sufficiently porous to permit an adequate passage of air, and this substrate characteristic is itself likely to change during the course of the fermentation. Evaporative cooling will also tend to dry out the substrate with time unless the air is humidified prior to entry into the fermenter, or if aeration is itself only used intermittently.

5. *pH control.*

Control of pH is reported to be a significant consideration in some scaled-up processes (Durand *et al.*, 1988; Lonsane *et al.*, 1992). However, in practise pH control may only be accomplished in processes that are agitated (to allow adequate mixing) and where moisture addition during the fermentation process can be tolerated. In all cases pH control is integral with moisture replacement (where evaporative cooling is practised) and acid or alkali is added to the water used to re-moisten the solids. Other processes are reported where strong buffering capacity is provided by the solids themselves (Lonsane *et al.*, 1985), and others where efficient buffering is provided at the start of the process by specific nutrient addition, e.g., by the use of urea as a nitrogen source (Durand *et al.*, 1988; Lonsane *et al.*, 1992).

6. Contamination control.

In many SSF processes, true aseptic conditions are difficult to maintain. This problem is exacerbated on scale-up, where larger volumes of material are being handled. Strategies that are normally adopted include the use of large inoculum ratios, moisture content control (to discourage bacterial contamination) and the use of various types of closed bioreactors or “covered” systems designed to prevent gross aerial contamination (Ahmed *et al.*, 1987).

7. Downstream processing.

Downstream processing is concerned with the further processing of the solids at the end of fermentation to obtain the required product(s). If the product is the fermented biomass itself, e.g. in the case of composting or up-graded animal feed, then little, if any further processing is required. However, if the product is an enzyme or microbial metabolite, then some form of extraction of the solids is required. A number of extraction techniques have been utilised such as percolation, multiple contact counter-current leaching, pulsed plug flow extraction, hydraulic pressing and supercritical fluid extraction (Lonsane *et al.*, 1992). Each of these methods has specific advantages and disadvantages. Percolation, for example, leads to a large volume of a very dilute extract (Ramakrishna *et al.*, 1982). Multiple contact counter-current leaching produces a more concentrated extract but is time consuming, laborious and requires skilled operators (Ghildyal *et al.*, 1991), and hydraulic pressing require a two stage process to give an adequate efficiency (Lonsane *et al.*, 1992). More recently, pulsed plug flow has emerged as the method of choice in some processes, particularly in India, since it

offers relative efficiency, simplicity of operation, economy, low capital expenditure and minimal labour involvement (Ghildyal *et al.*, 1991).

8. *Waste management.*

Unlike SLF, the volume of liquid effluent from a SSF process is generally low, the largest volume of waste consisting of the spent solids. The waste will potentially be highly polluting with typically a high BOD value and specific methods of disposal will normally be necessary. Of course in some processes, such as fermented food production and composting (where the fermented solids are the product itself), no waste burden is incurred. In other exceptional cases the spent solids can be put to further use. For example, in a SSF cellulase production process ensiling of the solids was shown to produce good quality animal feed (Roussos *et al.*, 1991). Other uses have included biogas production, composting and production of bricks, board and papers (Ghildyal & Lonsane, 1990). Much material will however inevitably go into land-fill with a high cost for transportation. In practise the disposal of solids at the end of a commercial scale SSF process is likely to add significantly to the overall cost, and can influence the final economic viability of a process.

1.5.4. Scale-up strategies.

Generally, the design of SSF systems has been largely empirical in nature (Durand *et al.*, 1996). The absence of accurate experimental data for SSF processes arises from the unreliability of many of the instruments used routinely for SLF when applied to low moisture conditions (Durand *et al.*, 1996). Further, the heterogeneous conditions

that prevail in SSF defy the straightforward characterisation of the growth kinetics of the organisms concerned. Direct growth data is inherently difficult to obtain in SSF since it is impossible to separate microbial biomass from the solid matrices. However, Zhu *et al.*, (1994) have described utilising polyurethane foam (PUF) as an inert carrier medium in a SSF system where they successfully obtained direct estimates of fungal biomass. Other indirect methods that have been attempted include following the development of fungal protein, DNA, ATP, ergosterol and glucosamine (chitin monomer) (Moo Young *et al.*, 1983).

Any attempt at mathematical modelling of an SSF system must first start with some method of quantifying the development of the fungal biomass. To use this data to produce a predictive model then requires the description of the relevant variables and their interactions. In SSF systems this becomes extremely complicated (Smits *et al.*, 1998). Figure 1.3 describes the major variables and the way they interact with each other in the course of a fermentation. The complexity is further compounded by the inherent heterogeneity of the system. In effect a multiplicity of “domains” will develop, each with a range of varying physical and chemical properties. To date no comprehensive mathematical model has been developed which can predict the outcome of a fermentation, or be applied to scale-up or process design. However, given the increased sophistication of computers, and the renewed attention that SSF is currently receiving, the development of a broadly applicable predictive model may only be a few years away (Smits *et al.*, 1998). To date, scale-up strategies in the development of commercial SSF processes have been largely confined to:

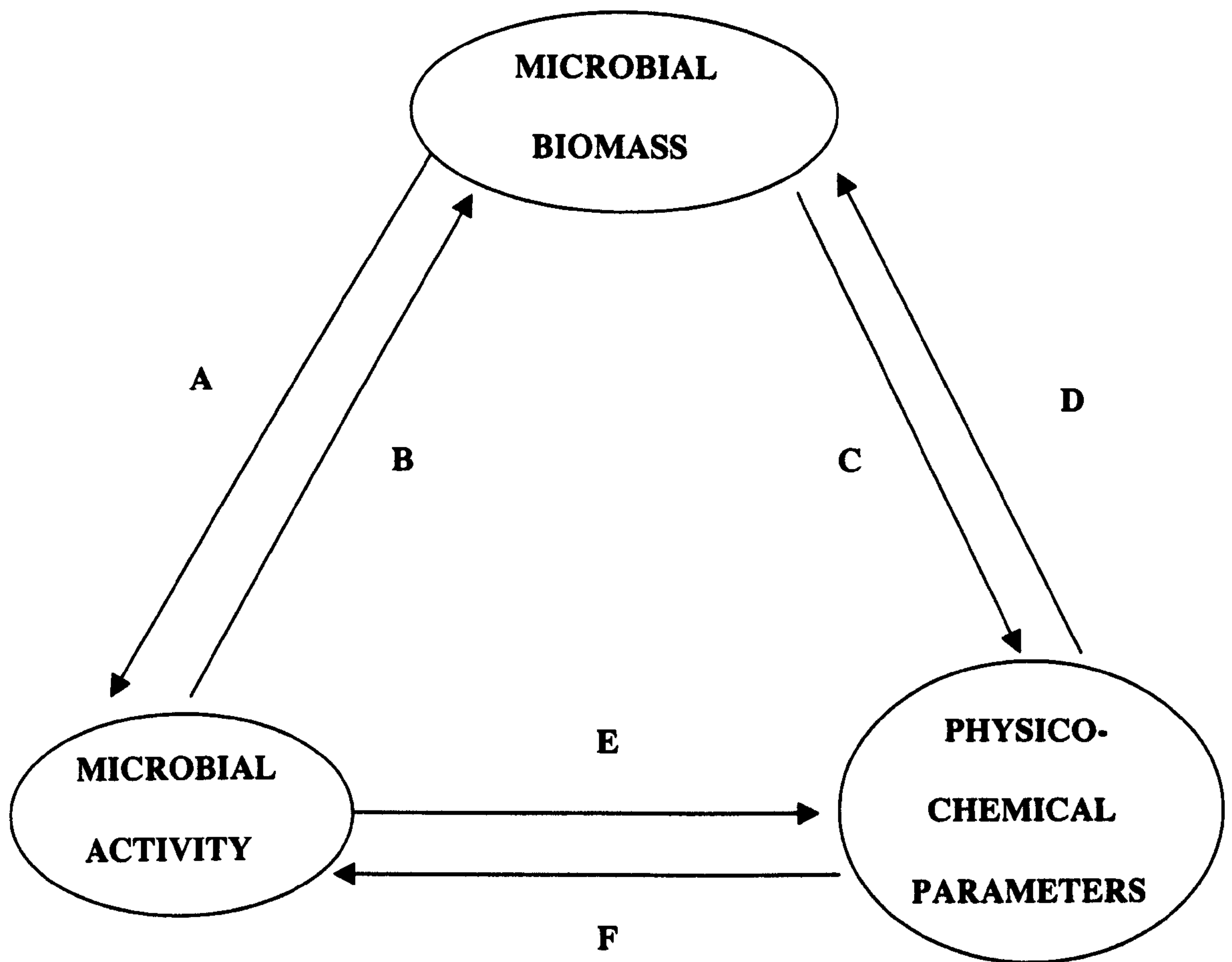


Figure 1.3. Qualitative dynamic model of a SSF system (adapted from Smits *et al.*, 1998). **A.** Biomass has a specific amount of activity. **B.** Microbial activity involves production of more biomass. **C.** Biomass influences physical properties of system e.g. void spaces of medium, a_w , compaction, diffusion and heat conduction. **D.** Changes in physical parameters influence development of biomass. **E.** Microbial activity influences chemical parameters of system e.g. CO_2 , H_2O increase, O_2 , nutrients decrease. **F.** Changes in chemical parameters influence microbial activity.

1. Trial and error (Lonsane *et al.*, 1992).
2. Maintenance of geometric similarity, based on conserving the same ratio of vessel dimensions from laboratory scale to industrial plant (Akao & Okamoto, 1983).
3. Maintenance of constant heat and water balances, based on conserving the same heat exchange and water levels as the process is scaled-up. In practice, this method probably relies on (1) and (2) above and can only be used in systems utilising forced aeration or some other method for heat and moisture control (Lonsane *et al.*, 1992).

1.5.5. Reactor designs

Historically, SSF has been confined to low technology applications such as traditional fermented foods, composting and (more recently) up-grading of agricultural waste products as animal feed. This is evident in the availability of SSF bioreactor designs, particularly for commercial scale processes, where the technological input often appears to be minimal. At their most simple, SSF processes consist of heaps of composting material kept in the open air. In the windrow composting process for example, solid waste is stacked in long rows 2m high by 3m wide and turned mechanically every few days (Moo-Young *et al.*, 1983).

The oldest application of SSF is the production of fermented foods. In the traditional manufacture of Koji the inoculated substrate (rice or soya beans) is distributed as a thin layer (5-7 cm) in small woven bamboo baskets. These are then stacked in tiers one above the other and separated by a gap of 10cm to permit aeration (Moo-Young *et al.*, 1983). This represents a type of thin layer, tray fermentation, which is the simplest form of SSF other than composting. Tray fermentation is often carried out statically, relying on the thin substrate bed to allow diffusion of gases and dissipation of heat. This limits the scale of most tray fermentation processes, scale-up often relying on simply increasing the number of trays used, which leads to a labour intensive process (Smits *et al.*, 1998). However, there have been some developments in tray fermenter design, leading to some quite sophisticated processes. In modern Koji fermentation for example, sterilised, heated and moistened air is pumped through the Koji “rooms” while rotating mechanical devices (augers) periodically turn the substrate bed. The process has therefore retained much of its traditional character but has achieved a high level of automation (Smits *et al.*, 1998).

More recently there has been development of closed drum type reactors which are designed primarily to mix the substrate, either continuously or intermittently, during fermentation. Two main types have been described:

1. Stirred bioreactors. In this design an external motor powers an internal shaft which turns augers, paddles or rakes (Durand *et al.*, 1996).

2. Rotating drum bioreactors. In this design the whole drum rotates on a series of rollers (Hesseltine, 1977; Durand *et al.*, 1996). At their simplest, cement mixers with little or no modification are utilised (Durand *et al.*, 1996).

Stirred or agitated type designs cannot be used in all applications due to the resulting shear damage to the mycelium which some fungal species cannot tolerate (Roussos *et al.*, 1993; Barrios-Gonzalez *et al.*, 1993).

The most sophisticated bioreactor design to date is the “packed bed” type. These most resemble SLF reactor designs since typically the substrate is housed in a cylindrical column sealed top and bottom during operation, with varying degrees of ancillary equipment. They are mainly confined to laboratory or pilot scale and have been used so far mainly for experimentation and feasibility studies. The research group at “Plate forme de Predevelopment en Biotechnologie-Inra”, Dijon, France have developed some of these designs to high levels of sophistication. They describe a number of designs for packed bed reactors all of which utilise forced aeration with sterilised, humidified, temperature regulated air (Durand *et al.*, 1993; Durand *et al.*, 1996). One system in particular, first reported by Gervais & Bazelin (1986) achieved temperature and moisture control of the medium during fermentation by means of the regulation of temperature and humidity of the air entering the reactor. Humidity of the inlet air was controlled by the temperature of the air humidifier unit, which in turn was controlled by an a_w probe in the medium. The temperature of this air was then set independently

by a separate heater unit controlled by a temperature sensor in the medium. Using this system temperature and water content of the medium were held within $\pm 2^{\circ}\text{C}$ and $\pm 2\%$ respectively, for a 50 hour “run”. Based on this design a larger reactor, with a total working volume of 1.6m^3 was developed which housed a substrate bed of 1m depth. This unit demonstrated similar process control but did not run aseptically (Gervais & Bazelin, 1986). Almanza *et al.* (1995) have described a pyrex laboratory scale reactor (1 litre capacity) which can be run aseptically after remote sterilisation of the substrate and transfer to the vessel. Chamielec *et al.* (1994) have described a stainless steel pilot scale fermenter (50 litre capacity) incorporating a screw type agitation device where medium sterilisation was achieved *in situ* by circulation of steam prior to fermentation. Bandelier *et al.* (1997) have described an aseptic, aerated and stirred packed bed design of 50 litre capacity for the production of gibberellic acid by *Gibberella fujikoro*i on wheat bran. This was a fed-batch type process with addition of supplemental feed stock (corn starch) after 72, 96 and 120 hours. A sterile sample handling device was also included which enabled aseptic sample collection during each run.

Some of the reactor designs developed by the Dijon group have found commercial applications. The first involves single cell protein production from agricultural by-products: In China two 25 tonne bioreactors have been constructed for microbial protein production using *Aspergillus tamar*ii. The second application has been developed as a collaboration with a French sugar manufacturer and involves production of microbial enzymes from sugar beet pulp, a waste product of the industry

(Durand & Chereau, 1988). The third application has been the production of a biopesticidal agent. In a patented process the entomopathogenic fungus *Beauveria bassiana* (an antagonist of *Ostriria nubilii*, the European corn borer) is grown on supplemented clay microgranules to produce typically 10^9 spores per gram of dry matter after 48 hours. The crude fermented product can be applied directly to the crop without further formulation. A specialist company has been set up and the biopesticide has been in production since 1994 (Durand *et al.*, 1996).

Another group, working in France and India, has produced a novel design of fermenter they have called “Zymotis” (based on the Greek word for fermenter). This was effectively a packed bed forced aeration design of 100 litre capacity where metabolic heat removal was accomplished by a series of water cooled heat exchanger plates, arranged so as to divide the working volume up into a number of compartments. This design performed well in terms of fungal enzyme production (cellulase) over a range of substrate loads (13-40kg moist medium) (Roussos *et al.*, 1993).

Fasidi *et al.* (1996) described a tunnel type reactor used mainly for the production of edible mushrooms where air flow and temperature were monitored and maintained by computer control. This design of reactor also allowed for a degree of automation in operation.

There have been some limited reports of air-solid fluidised bed type fermenters in the literature (Roussos *et al.*, 1993). One particular example was a gas-solid spouted bed bioreactor which was used for amylase production on rice by *Aspergillus oryzae*. In this design intermittent spouting of the substrate was used in place of aeration and agitation, and gave good results compared to static and packed bed (low aeration level) fermentations. However, this design was developed only at small scale (200g of moist medium) and was not run aseptically (Silva & Yang, 1998). Obvious limitations of these systems are the huge air and energy requirements to keep particles in the turbulent state, and the high level of technological sophistication required.

1.5.6. Applications.

A body of literature concerned with the modern application of SSF is now developing. Tengerdy (1996) has described the use of SSF for the production of cellulase. He found that SSF was a quicker route, leading to a more concentrated product which could be used directly in the crude form. The area of protein enrichment of agricultural wastes and by-products seems to be particularly active and is reported by other workers (e.g. Nigram and Singh, 1996). Soccol (1996) has reviewed the potential of cassava root wastes as a substrate for production of enzymes, citric acid and edible mushrooms by SSF. There has been recent interest, mainly in India, in the production of several antibiotics and other secondary metabolites using various solid agricultural wastes and residues as solid substrates. These include penicillin, cephalosporin, tetracyclines, cyclosporin-A, iturin, ergot alkaloids, gibberelic acid and various mycotoxins. All of these substances were successfully produced in SSF, and

in many cases there were significant advantages over the more conventional SLF such as higher yields, quicker production, lower energy requirements and simpler waste management (Balakrishnan and Pandey, 1996). Barrios-Gonzalez and Tomesini (1996) have reviewed in detail the production of aflatoxins in SSF. They have reported extremely favourable yields of aflatoxin from SSF compared to SLF and described catabolite repression and feedback regulation mechanisms operating in the same way as for SLF. Larroche (1996) has also described the use of SSF for the production of spores of various fungi (e.g. *Penicillium roquefortii* and *Aspergillus ochraceus*) which are used in a number of biotransformation processes.

1.6. NATURAL PRODUCTS – THEIR PLACE IN MODERN DRUG DISCOVERY.

1.6.1. Introduction.

The use of natural products (secondary metabolites of microbial, plant or animal origin) stretches back into antiquity. Ancient civilisations in India record use of plant products to treat diseases from as early as 1000 BC, and in China there are records of the use of such products from 500 BC. The Ancient Egyptian Ebers papyrus (1550 BC) documents several active ingredients that are still in use today (Harvey, 1992).

But what place do natural products have today? Interest in these as a source of new drugs undoubtedly declined recently due to the advent of new technologies. In fact as far back as the 1960's the pharmaceutical industry as a whole went through a period

of disillusionment with natural products considering them “too difficult” and “all the exciting molecules already discovered” (Demain, 1999). However, as recently as 1990, of the 20 top selling drugs in the world, four were derived directly from natural products, and two others originated from naturally derived actives (Harvey, 1992). In 1992, of the 43 new chemical entities to be introduced 18 originated directly or indirectly from natural products.

1.6.2. New Technologies.

A number of new technologies have grown up in recent years which have relevance to drug discovery, and were thought, by some, to be modern replacements for natural product based discovery programs. The most obvious example is combinatorial chemistry. Combinatorial chemistry is a technique which produces “libraries” of related compounds built by coupling pools of monomer sets. For example, a peptide tetramer library of the 20 naturally occurring amino acids will give 160,000 different molecules that can be used in screening programs (Domanico, 1994). Combinatorial techniques can therefore produce diverse libraries of compounds inexpensively with straightforward purification and identification. Other relevant techniques include computer aided rational drug design and biotechnology itself (Williams *et al.*, 1993). One of the most recent emergent technologies is pharmaco-proteomics which is concerned with obtaining extremely detailed protein profiles of various cell types, either healthy or in disease states. This technology is used to select drug targets (proteins associated with specific diseases), develop new drug candidates rationally and validate them rapidly (Ashton, 1999).

1.6.3. Modern approaches to natural products

Natural products, when selected in screening assays rarely become drugs themselves but they may serve as “suggestions” or templates for therapeutic agents. A notable recent exception to this is *Taxol*, derived directly from the Pacific or Western Yew tree (*Taxus brevifolia*) which has found direct application for the treatment of refractory ovarian cancer. In higher plants, the belladonna alkaloids (e.g. atropine), quinine, cocaine, certain opiates and salicylic acid have all served as models for the design of anticholinesterases, antimalarials and analgesics such as pentazocine, methadone and aspirin, and many others (Balandrin *et al.*, 1993). It is not within our ability yet to routinely produce such lead molecules from a knowledge of the molecular structure of disease targets – we need nature to present us with the initial ideas (Caporale, 1994).

Chemical ecology is one approach to modern drug discovery which is centred on natural products, and seeks to understand the biochemical basis of ecological interactions between organisms. One simple example of this type of approach is the study of the leaf cutter ant, *Atta cephalotes*. This ant collects leaf cuttings which are used to cultivate a particular fungus as a food source. Careful observation of the ant has revealed that it avoids leaves of the plant *Hymenaea courbaril*. These leaves have subsequently been found to contain a powerful terpenoid which strongly inhibits growth of the cultivated fungus. Therefore observation of the behaviour of this ant, and in particular the plant species avoided, could in principle lead to the discovery of

novel antifungal agents (Caporale, 1994). Implicit in the whole approach of chemical ecology is the maintenance of biodiversity since, as species are lost, the complex links with other species are also lost forever. This problem is greatest in the tropics, particularly in the rain forests. These regions contain most of the worlds plant species and more than half of these (and their associated micro-flora) are still unknown to science. In tropical Latin America, including Brazil, the rate of decimation of rain forest currently guarantees that significant opportunities for drug development will be lost forever (Caporale, 1994). In recognition of this, a number of large pharmaceutical companies, notably Merck, Glaxo Wellcome and Smithkline-Beecham are sub-contracting natural products research in the tropics and supplying funding for local conservation projects.

Genetically engineered micro-organisms are beginning to be employed in the production of “new” natural products. In particular, recombinant actinomycetes have been used to produce new aromatic compounds and analogues of macrolide antibiotics that cannot be produced by synthetic chemistry (Hutchinson, 1994). Recombinant organisms have also been employed to express genes from “unculturable organisms” (e.g. from DNA isolated directly from soil, or from fungi present in lichens). This genetically based approach combines natural products research with biotechnology and is viewed as a promising area for future drug discovery.

1.6.4. Comparing natural products with synthetic chemical development.

There is still much debate regarding the route that future drug development programs are likely to take, and the relative importance of natural products and synthetically derived chemicals in these programs. Natural products have the advantage of being the result of millions of years of diverse organic chemistry directed by natural selection. The resulting structures are hugely diverse themselves, often described as “unusual” and “imaginative”, and rich in stereochemistry and reactive functional groups (Demain, 1999). As products of natural selection they are likely to be “tough”, resilient molecules with a high degree of structural stability. By definition, they will demonstrate biological activity in the right assay system (i.e. they are biologically “relevant”) and they will typically show a reasonable degree of solubility and bioavailability. In contrast, synthetic chemicals, and particularly combinatorials, are purely the result of random additive chemistry experiments and therefore lack the inherent diversity of natural products as well as the biological relevance (Mocek *et al.*, 1999).

There are however some problems inherent with natural products. They are often obtained in the form of complex mixtures of non-uniform concentrations making isolation and identification time consuming and difficult. They may present significant challenges for production on a commercial scale, particularly in the case of fermentation products, and they may prove to be impossible to produce via a synthetic route (Mocek *et al.*, 1999). Also, source material for natural product isolation may

become difficult to obtain in the future due to biodiversity convention ambiguities, and the general loss of biodiversity worldwide.

1.6.5. The future of drug development.

The discovery of new chemical entities that may be developed into drugs is now based on assay systems that combine robotic machinery with computerised data analysis. These systems are capable of processing tens of thousands of samples each year (at least) so that successful drug discovery depends to a large extent on the supply of new and novel chemicals that can be screened in these systems. It seems likely then, that future drug discovery will depend on a combination of all the techniques available to supply the required “feedstock”. Natural product programs will certainly contribute as one of these techniques, either directly (as in the case of modern products like *Taxol*), in the form of recombinant products derived from biotechnology based programs, or by supplying starting points or “ideas” for synthetic analogues. In short, natural products continue to be very important. Arnold Demain summed-up the situation most succinctly at a recent meeting of the Royal Society of Chemistry (St. Andrews, Scotland, Sept. 1999):

“Natural products research comes and goes but will always come back. What’s needed now is a blend of the old and the new. We are still only at the beginning”.

1.7. OBJECTIVES OF THE PROJECT.

This is a “Total Technology” PhD project which is designed as a collaborative venture with an industrial sponsor company, and is intended to address an area of particular interest to the company. This project was carried out in collaboration with TerraGen Discovery Ltd., Slough UK., a company that routinely uses SSF techniques in drug discovery and development programs. This project has been designed to answer specific questions about some of these practices, in particular scale-up methods. As such, the project is of an “applied” nature. The project includes a separate “non technical” section on a relevant related topic.

The specific project objectives are as follows:

1. To research and write a review of the modern Pharmaceutical industry to form the “non technical” chapter of the project.
2. To evaluate the effects of interactions between substrate type (using extracts of solid substrates), water availability and temperature on growth and secondary metabolite production, on solidified agar surface cultures of a *Phoma* species. This study was designed as a “lead in” to subsequent work with solid heterogeneous agricultural substrates.
3. To prepare moisture sorption isotherms for a number of solid substrates, for the first time, in order to accurately control water availability in subsequent studies.

This included both simple single substrates as well as composite, “two phase” systems.

4. To investigate the effects of substrate type and water availability on growth and secondary metabolism of (a) *Epicoccum nigrum*, (b) *Sarophorum palmicola*, (c) *Drechslera dematioidea* and (d) *Corynespora cassiicola* with a view to increasing the titres of certain metabolites of pharmaceutical interest. This information was then used to select a fungal species/substrate combination to use as a model system for further work (*E. nigrum* and bulgar wheat ultimately chosen).
5. To determine the key physical and environmental parameters involved in a model solid substrate fermentation system and their relationship with secondary metabolite production using *E. nigrum*.
6. To determine the critical physical and environmental factors influencing scale-up of a model solid phase system for reproducible metabolite production, using *E. nigrum* and comparing metabolite profiles with those obtained in small scale studies.
7. To develop prototype scale-up procedures to a level of several litres of wet substrate, for production of satisfactory levels of metabolites in a model solid phase system.

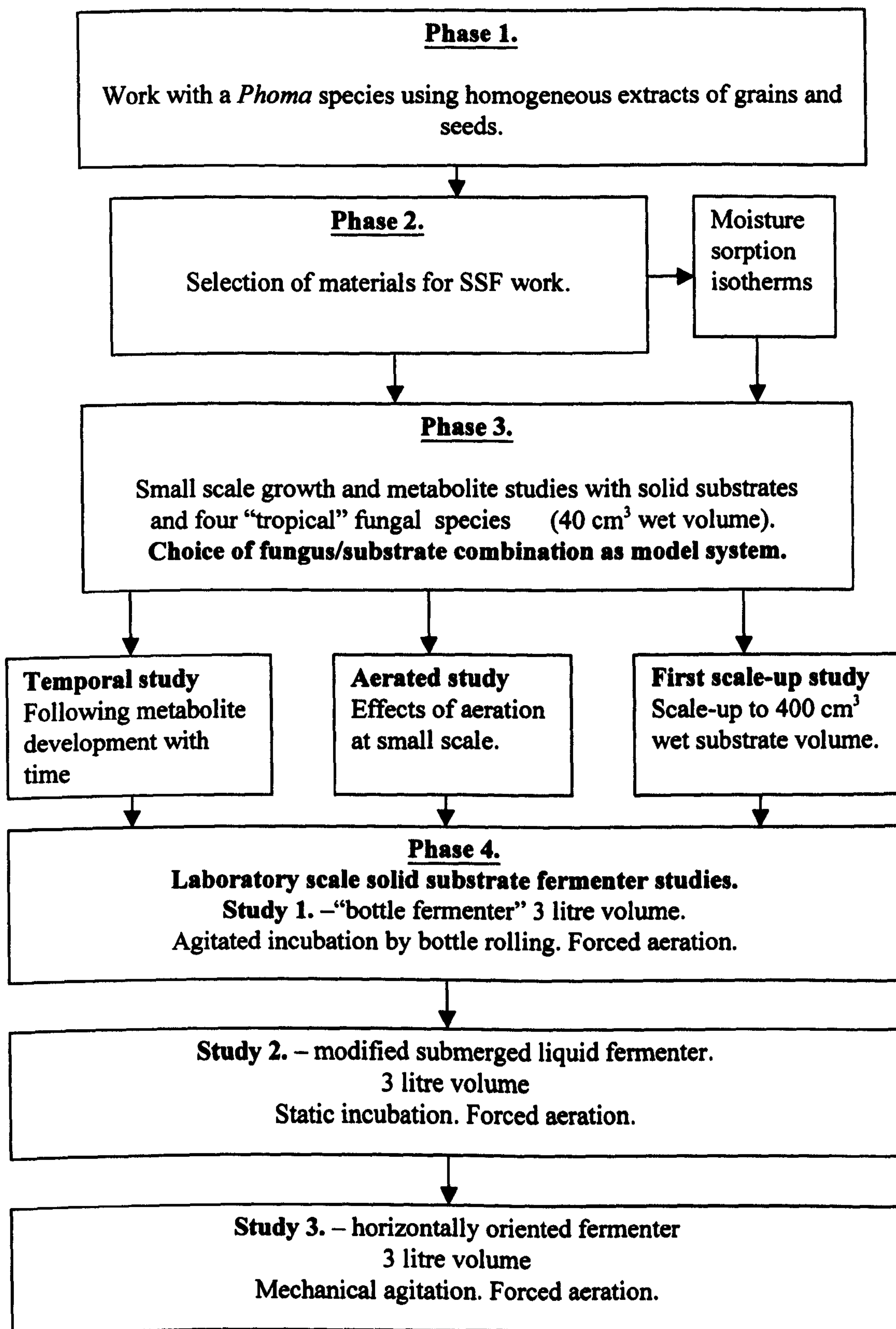


Figure 1.4. Schematic flow diagram of phases of work in the project.

CHAPTER 2

NON-TECHNICAL CHAPTER

THE MODERN PHARMACEUTICAL INDUSTRY – CHANGES, PRESSURES AND NEW INNOVATION.

2.1. Introduction.

“Well, in our Country” said Alice, panting a little, “you’d generally get somewhere else - if you ran very fast for a long time, as we’ve been doing”.

“A slow sort of Country!” said the Queen. “Now here, you see, it takes all the running you can do, to keep in the same place”.

In Lewis Carroll’s 19th Century story “Alice through the Looking Glass” the Red Queen (a “life size, animated” chess piece) gives voice to a literal interpretation of the concept of “running to keep still”, or expending all of one’s efforts just to maintain the *status quo*. Evolutionary biologists today often take the “Red Queen’s race” as a metaphor for the unremitting war we need to wage against infectious disease. Many believed the war to be won, with the advent of the antibiotic era, but this may yet prove to be just a lull in hostilities as antibiotic resistance begins to become commonplace in many microbial species (Lenney, 1999). In 1997 the Select Committee of the House of Lords on Science and Technology carried out a Public Inquiry into the problem of the development of resistance to antibiotics and antimicrobials. The report was published in April 1998, and the Committee was reportedly “shocked and dismayed” by it’s findings (Desselberger, 1998). In addition, the World Health Organisation (WHO) recently announced : “ Too few new drugs are being developed to replace those that have lost their effectiveness. In the race for

supremacy, microbes are sprinting ahead". And this situation - fuelled as it is by the generally complacent misuse of antibiotics that are over-prescribed for everything from stubbed toes to viral colds (and used freely in animal production) - is not the only health threat to the human race. The AIDS virus continues its all-but unchecked spread in many parts of the world, while tuberculosis, malaria and other serious diseases such as cancer continue to claim millions of lives each year.

So, on the face of it, there would seem to be plenty of incentive, even moral obligation for the pharmaceutical companies to go all out in their innovation programs to find new therapeutic compounds to stay "ahead of the race". But how is the modern pharmaceutical industry positioned to address the threats and challenges of present day disease? What other pressures and constraints are likely to impinge on the innovative process? What technologies and tools are they most likely to utilise in these programs? To attempt to answer these questions it is necessary to look a little closer at the pharmaceutical industry as a whole.

2.2. The Pharmaceutical industry – historical perspective.

The pharmaceutical industry plays an important role in society, although this has sometimes been claimed to be exaggerated beyond its true value. This importance results from its perceived joint responsibility with the medical profession for the safeguarding of health. However, the industry is not without its critics: There have been those who have accused it of "unadulterated greed" and even "profiteering on the misery of others" (Craig & Malek, 1995).

As a whole, the pharmaceutical industry addresses two major markets: the intramural market (hospitals) and the extramural market (medicines used at home). The extramural market is by far the largest. In principle, the way the pharmaceutical industry operates within these markets is quite simple: A company innovates, finds a new drug, takes out a patent, markets the innovation, recovers the research costs and makes sufficient profit to do more R&D. Meanwhile the patent on the drug runs out so that price competition comes into play, i.e. the drug can be copied at low cost by “generic” manufacturers (Craig & Malek, 1995). One complication in this simplistic model comes from government intervention. Independent government bodies, e.g., the Federal Drug Administration (FDA) in the US, closely regulate the industry and demand rigorous testing of new therapeutics before they can enter the market. This intervention increases the cost of R&D, and, by introducing delay before approval, erodes the patent life of a drug (Craig & Malek, 1995).

Despite these constraints on profit level, the pharmaceutical industry has been extremely successful historically, and by the 1970's had grown into a highly abnormal global business. There were some 7000 companies, all prospering, none of which had ever gone out of business, despite an average spending of 10-15% of sales profit per year on high-risk research (James, 1994). This situation was based mainly on low price sensitivity, a significant knowledge gap between the manufacturer and the customer, and a passive customer base. The pharmaceutical companies sold, at premium prices, anything they could discover. Neither was there a great threat of

competition, because this was based not on price but on the ability to introduce new products, all at high prices (James, 1994)). It was even possible for clinically indistinguishable products to be introduced successfully at higher prices than established drugs. These “me too” products (as they are often called) have been a major source of revenue historically for the industry. For example, between 1989 and 1993 the FDA approved 127 new drugs of which only a small minority offered a clear advantage over existing therapeutics. The preponderance of “me-too” products seems to have arisen because different pharmaceutical companies tended to develop similar drugs concurrently, and also attempted to win market share where there was already a clearly established “breakthrough” product, since even a very small market share meant large revenues (Kessler *et al.*, 1994).

In many cases companies introducing a new product would rely on the premise that “newer is better” and therefore worth more (Kessler *et al.*, 1994). Aggressive advertising and a lack of knowledge amongst customers facilitated this view. Some companies have been accused of entering into more unethical practices to promote these types of drugs. Of particular concern to the FDA have been reports of “seeding trials” where companies sponsored studies of little or no scientific value simply to be able to recruit “investigators” (usually practising physicians) to prescribe the drug under trial, for which they received lucrative payments. These “studies” represented thinly disguised promotional campaigns. Another practice of concern that the FDA has on record was the making of false and misleading claims for unremarkable products: Advertising campaigns were mounted based on no supporting data, or

relying on the absence of data. Negative claims surrounding competitors products, based on the selective use of data, were also made. The FDA also reported “switch campaigns” where patients were induced to switch from their usual drug to a “me-too” product. This practice involved paying pharmacists to encourage them to influence the prescribing habits of the physicians they supplied. “Switch campaigns” were often based on claims of superior efficacy and/or lower cost, but often the medical implications of therapeutic shift were not considered carefully enough, and the cost argument was not always substantiated (Kessler *et al.*, 1994).

2.3. Changes in the Pharmaceutical Market.

The result of all of this was that, from the 1950's to the 1970's, the pharmaceutical companies enjoyed a charmed life, with uninterrupted growth unparalleled by any other global business (typically >20% increases in annual sales and profit). The industry enjoyed a continuous demand for more and better health care and a limitless public and private finance for prescription drugs. However, there were some early warning signs of change, mostly ignored by the industry, as far back as the 1970's. At this time, in a climate of recession, the economic problems of meeting the spiralling healthcare costs of an ageing population with fewer tax-payers (typically 10% unemployment at this time) were becoming clearly visible (James, 1994). And by the early 1990's the previous certainties that had held the industry in its privileged position had been swept away.

1993 was a particularly crucial time for the industry. German healthcare reforms in January and the Clinton proposal for the American Health Security Act in October of that year signalled an end to increasing costs of healthcare that would be tolerated by governments. In the UK too, a new Pharmaceutical Price Regulation Scheme (PPRS) was introduced, to run until 1996. And, although the Clinton administration proposals had stalled somewhat by 1995, these events heralded a global shift in customer behaviour towards the pharmaceutical industry (James, 1994). Public and private buyers began to pursue cost containment and decision making power moved steadily upstream to coalitions between insurers and hospitals, and wholesalers and retailers. The growing personal interest in healthcare forced a growth in patient decision making. A shift occurred, particularly in the US, to the management of the delivery of healthcare by maximising health benefits at the lowest possible cost, termed “managed care”. This increased sophistication and concentrated buying power forced attention generally on “value for money” and “added value” rather than product technology. In short, the real power began to shift away from the manufacturer and onto the customer (James, 1992), and two new “realities” became clear to the industry:

1. Price flexibility had disappeared from all major markets.
2. Annual sales and profit growth of greater than 20% was a thing of the past.

(James, 1994).

By 1994 price-wars dominated the selling of drugs. For example, Sandoz's *Lescol*, introduced in 1993 and the fourth cholesterol lowering drug on the market, was selling at half the price of the therapeutic class leader, *Mevacor* (Merck & Co.). The other remaining competing drugs: Pfizer's *Zoloft* and Smithkline Beecham's *Paxil* sold for 15% less than the Merck & Co. product at that time (Weber, 1994).

2.4. Pressures on R&D.

Around the same time a third uncomfortable "reality" had become clear, namely that there was no longer any guarantee that R&D funding would produce any return on investment. Most pharmaceutical companies need to sustain annual growth of around 10% to maintain their R&D effort. In the previous favourable economic climate this level of growth had been easy to achieve and R&D had developed into a rather inefficient, "affordable luxury" in most of the companies. The total cost of bringing a new product from discovery to launch has been estimated at between \$255 m - \$369 m, with 90% of this centred around product development (James, 1994). The traditional screening approach has itself been described as "a large scale adventure in serendipity - laborious, inefficient, costly and conceptually inelegant, and dependent on technology founded in the 1960's and 1970's" (Sun and Cohen, 1993). This technology was made to look even more outdated by the emergence of biotechnology based therapies in the 1980's. In many companies spending on R&D had been poorly controlled with often a growth in facilities but no real improvement in innovation. Regulatory demands (and therefore costs) have risen steadily since the 1960's, fuelled in part by the tragedy of thalidomide which brought about new controls and caution in

the industry (James, 1994). These additional regulatory demands have, in some instances included the introduction of pharmacoeconomic studies. Pharmacoeconomics is a relatively new discipline that identifies and evaluates the comparative costs and consequences of pharmaceutical products and services. It has been defined as “the description and analysis of the costs of a drug therapy to healthcare systems and society”(Milne, 1994). This new regulatory area is a direct consequence of the new sophistication and cost awareness of the pharmaceutical customer base and means that, for the first time, a new therapy may be refused approval on economic grounds alone. Not surprisingly, pharmacoeconomics is generally unpopular with the industry and has been described on occasions as “a discipline with no discipline” (Milne, 1994). Against this backdrop of increased regulatory requirements, the inherent complexity of innovation has itself tended to increase due to a general decline in the number of useful new products discovered. This is because the most successful drugs had previously targeted long-term chronic diseases in large numbers of patients (cardiovascular, gastrointestinal and musculoskeletal conditions) (James, 1994). By the 1990’s there were few opportunities left in these areas since satisfactory low-cost generic products were available for these conditions and the new economic pressures made the pursuit of more “me too” products non-viable. Up to 1994 the development pipeline in innovation still contained some derivative products, but these would have come under intense scrutiny at this time and virtually all would have been abandoned by the middle of the decade. The major remaining unconquered diseases, such as AIDS and

cancer, and perhaps also the threat of wide-scale antibiotic resistance, represented far more difficult problems for developing effective remedies.

2.5. Responses of the Pharmaceutical Companies.

So, by the early 1990's the pharmaceutical industry has seen an almost complete reversal in it's fortunes. It's market had transformed from one consisting of a passive, price insensitive customer base, without the skills or motivation to compare efficacy and value, to one that was knowledgeable, sophisticated and value driven.

These fundamental changes in the pharmaceutical industry signalled a number of restructurings of companies and policies, the most dramatic being consolidation. In the first 16 months from August 1993, 11 major companies were involved in acquisitions. The trend continued at least for the next 3 years. In 1996 a "flood" of take-overs and mergers was still occurring, some of which were hostile, as the industry started to take on a predatory nature. One notable example was Glaxo's hostile take-over of Wellcome for £8900m in 1995 which resulted in the wholesale closure of Wellcome's R&D laboratories at Beckenham, Kent. Other major acquisitions have been SmithKline Beecham's purchase of Stirling Winthrop (1994), Roche's purchase of Syntex (1994), the merger of Pharmacia with Upjohn (1995) and Rhone-Poulenc Rorer's take-over of Fisons in 1995 (Hook, 1996). This type of activity was, and still is, designed primarily to acquire greater market share, cut overlapping R&D costs and enter into closer customer relationships.

Some of the companies began to target markets that were new to them. By 1995 Hoechst, Ciba and BASF had entered into the generics market, and the previous bitter rivalry between these two sectors appeared to diminish as both groups began to explore new types of relationships with each other (Archer, 1995). Other companies began to look at the “over the counter” (OTC) market, particularly in areas such as smoking cessation aids, antivirals (e.g. “cold sore” remedies) cough/cold remedies and topical antibiotics (Archer, 1995). Other types of re-structuring exercises involved looking closely at internal practices; Merck & Co. have re-engineered their manufacturing processes with significant savings, and achieved a reported 50% improvement in productivity. Other companies have focused attention on improving the development time of a new drug so that the time to launch is reduced and the patent protection period increased (James, 1994). Of course, most of this activity involved radical re-structuring of the companies concerned, often called “downsizing” or “delaying”, both euphemisms for wholesale redundancies.

While many companies were becoming involved in mergers and acquisitions, there was also, for the first time, a steady stream of companies moving out of the industry. Examples of companies pulling out at this time are Air Liquide in France, Kodak in the US and Boots in the UK. Also notable was a lack of new “players” entering the industry. Any new start-up companies were likely to be integrated early on into one of the emerging “giants”.

Movement closer to the customer became a key objective for the more far-sighted companies. The intention was to develop into a seamless, integral part of the whole healthcare chain, to nurture an image of understanding of customer needs and a willingness to take responsibility for every aspect of disease management. In short, to demonstrate a realisation that people wish to be healthy rather than to be given drugs (James, 1994).

2.6. Changes in R&D.

Not surprisingly, R&D effort has come under close scrutiny. At its simplest, companies have adopted a more rational approach to R&D by re-organising and streamlining their effort and abandoning anything which looked like a “me too” product. Other companies have made more sweeping changes. In 1994, Eli Lilly & Co. abandoned hypertension, pulmonary and virology research to concentrate solely on central nervous system and cancer research. At the same time Zeneca in the UK halved the therapeutic areas they were working in. Other companies have made significant inroads into the biotechnology sector, mostly in the form of strategic alliances with existing biotechnology companies. For example, in 1995 Ciba was working with Chiron on optical gene mapping, and with Neurospheres on regeneration of brain cells. Hoffman-La Roche was investigating genomic therapies for obesity and non-insulin dependent diabetes with Millennium Pharmaceuticals. Rhone Poulenc and Smithkline Beecham were the companies most heavily involved in biotechnology research, with the latter applying two-fifths of its research effort in this area, and the former setting-up the subsidiary company Gencell which was

designed to develop a network of collaborations with other companies (Walters, 1995). In fact, as early as 1990, biotechnology derived pharmaceuticals and diagnostics were beginning to arrive on the market, although some of the biotechnology start-up companies of the 1970's and 1980's had themselves witnessed hard times. Some had been acquired early on by pharmaceutical companies (e.g. Genentech by Hoffman La-Roche) while others had disappeared completely by this time (Fishlock, 1990). By 1993 there were 16 "biologics" (drugs based on recombinant DNA technology) commercially available, 16 more in clinical trials and an estimated 120 awaiting clinical trials. However, not all of these new therapeutic agents were ultimately able to demonstrate significant advantages over established drugs, and undoubtedly suffered the same fate as the more conventional "me too" products. Others failed to demonstrate efficacy in clinical trials. Many of the recombinant DNA products suffered from difficulties with bio-availability and side-effects, and some were very expensive. Genzyme's recombinant treatment for Gaucher's disease, for example, had the dubious honour of being the most expensive drug in the world, with an annual treatment cost of \$765,000. There were some clear winners, however. Two examples are Genentech's treatment for cystic fibrosis and HybridTech / Lilly's recombinant human insulin (Williams *et. al.*, 1993).

There have been other changes in R&D: Some companies have adopted sophisticated econometric tools to evaluate the risks and potential of R&D. These tools include a number of elaborate mathematical models such as "Monte Carlo Analysis" and "Options Theory".

Monte Carlo Analysis is used to predict the effects of simultaneous changes in variables such as scientific viability, marketing, manufacturing and economics. Options Theory, borrowed from the finance industry, is concerned with evaluating investments (Houlding, 1995). Some companies have gone even further: Smith Kline Beecham, for example, has begun to impose “market aligned planning” into their R&D effort. This means that when a drug candidate has demonstrated potential on scientific and economic grounds, the R&D department enters into a contractual relationship with the marketing department concerning the drug that will be produced (Houlding, 1995). “Market aligned planning” is indicative of a shift in the importance of marketing relative to R&D. Marketing is now the central business philosophy of the industry. R&D has become marketing led in an effort to produce competitive new products tailored to the demands of the new sophisticated customer base (James, 1992).

2.7. The Future.

So, at the start of the new Millennium, the pharmaceutical industry finds itself operating in a new aggressive and unforgiving market place. Not a single company has been untouched by these changes. Most have sought some form of strategic alliance, others have disappeared. All have seen their profit margins eroded. But, to return to our initial questions, where does this leave the surviving companies in terms of dealing with the significant diseases of today? We have seen that R&D, in its old form, has been one of the areas hardest hit by the economic changes in the industry.

And yet, R&D, and the discovery of effective new therapies is still the essential lifeblood of the successful pharmaceutical company. Weisbach and Moos (1995), writing in *Drug Development Research* set out a clear “prescription” for future success for pharmaceutical companies in the new economic climate. They recognised the need for continued R&D effort, and made the following recommendations:

1. Rapid expansion into biotechnology based effort, either by contractual arrangement or acquisition, leading to 50% of total effort in this field.
2. The further re-defining of R&D effort into clearly focused, truly innovative areas.
By this the authors probably have in mind such new enabling technologies as computer assisted design, combinatorial chemistry and pharmaco-proteomics, the latter being a significant emergent technology in the late 1990's (Ashton, 1999).
3. The maintenance of specialist skills such as high volume, large scale organic synthesis, large animal research laboratories, toxicology and natural products fermentation capabilities.

The “prescription” for success therefore seems to rest squarely with continued R&D effort, but that which is more clearly focused and incisive. The incentive to continue to engage in R&D is very clear. Indeed it is, more so than ever, the incentive of survival. We may conclude therefore that the pharmaceutical companies of the new Millennium are well placed to engage in the “Red Queen’s Race”, using a whole

battery of methods and technologies, some of which are old, and some new. R&D appears to be alive and well with most companies still allowing, in part of their effort, “a relatively free association of its scientists who are allowed to range far and wide to follow their whims”. Cecil B. Pickett, executive vice president of Schering – Plough has said (Weber, 1994):

“You must give people the opportunity in the laboratory to use their own judgements and instincts, and the serendipity will come”.

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CHAPTER 3

MATERIALS AND METHODS

3.1. MICRO-ORGANISMS USED IN THE PROJECT.

3.1.1. Initial *in vitro* work with grain extracts.

1. *Phoma* species (IMI 332962).

The organism used in the *in vitro* part of this project was a *Phoma* species, originally isolated from soil in *Armaco de Pera*, Portugal and donated by Glaxo Research and Development, Stevenage. It could not be grouped within an existing species of *Phoma* by CABI Biosciences (Egham, Surrey. UK.). Typical morphology of the isolate on malt extract agar at 25°C was a “smoky” grey “low” mycelium (see Plate 3.1a). This organism is of particular interest since it produces members of a class of secondary metabolites called squalostatins (or zaragozic acids). Since 1991, eleven fungal taxa have been found to produce this family of metabolites, perhaps indicating an important natural role in the “lifestyle” of these organisms. This class of compounds came under intense scrutiny in the early 1990’s when it was discovered that they act as potent inhibitors of cholesterol and ergosterol biosynthesis via competitive inhibition of squalene synthase (Dawson *et al.*, 1992; Bergstrom *et al.*, 1995). Squalestatin 1 (S1), the principal metabolite produced by the organism used in this research was found to be active against mammalian, yeast and fungal synthases (mammalian synthases tested in rat liver homogenates). More importantly, *in-vivo* tests involving marmosets (which exhibit lipoprotein metabolism similar to that of man) gave 75% reduction in serum cholesterol levels (Baxter *et al.*, 1992). However, S1 subsequently failed toxicity trials carried out by Glaxo R & D and therefore was not developed for drug use. This isolate however represents a very useful model

system for research, providing a secondary metabolite with direct pharmaceutical relevance. It has previously been shown to respond strongly, in terms of S1 production, to fluctuations in water availability in both solidified (agar) and solid substrate culture (Baxter, 1997; Baxter *et al.*, 1998).

3.1.2. Tropical species used with solid substrates.

2. *Corynespora cassiicola* (Berk & M. A. Curtis.) C. T. Wei. (Plate 3.1.b)
3. *Drechslera dematiodea* (Bubak & Wrobl.) Subram & B. L. Jain. (Plate 3.1.c)
4. *Sarophorum palmicola* (Henn.) Seifert & Samson. (Plate 3.1.d)
5. *Epicoccum nigrum* Link. (Plate 3.1.e)

These species were received from TerraGen Discovery Ltd. (formerly Xenova Discovery Ltd) in October, 1997. These organisms were isolated from the following materials: 2; soybean plant, 3; maize plant, 4; waterlogged palm fruit in rainforest swamp, 5; leaf vein of unidentified plant.

These organisms were selected for use in the project since they all have well characterised metabolite profiles when grown on the TerraGen solid substrate system. Organisms 2, 3 and 5 above are known plant pathogens requiring a MAFF Plant Health License.

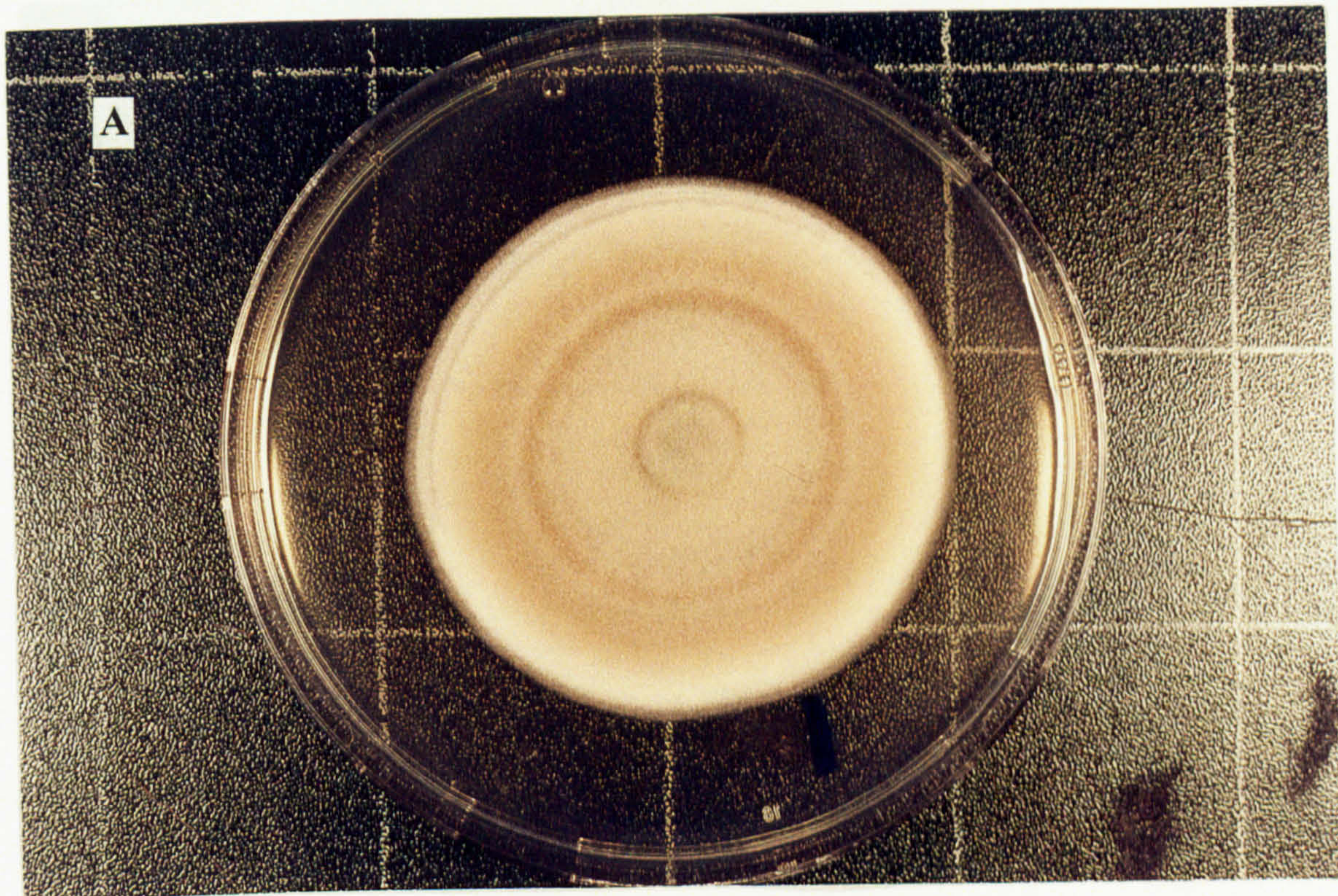


Plate 3.1. Morphology of fungal isolates on malt extract agar (MEA) (2 week incubation, 25°C). A. *Phoma* species. B. *Corynespora cassicola*.

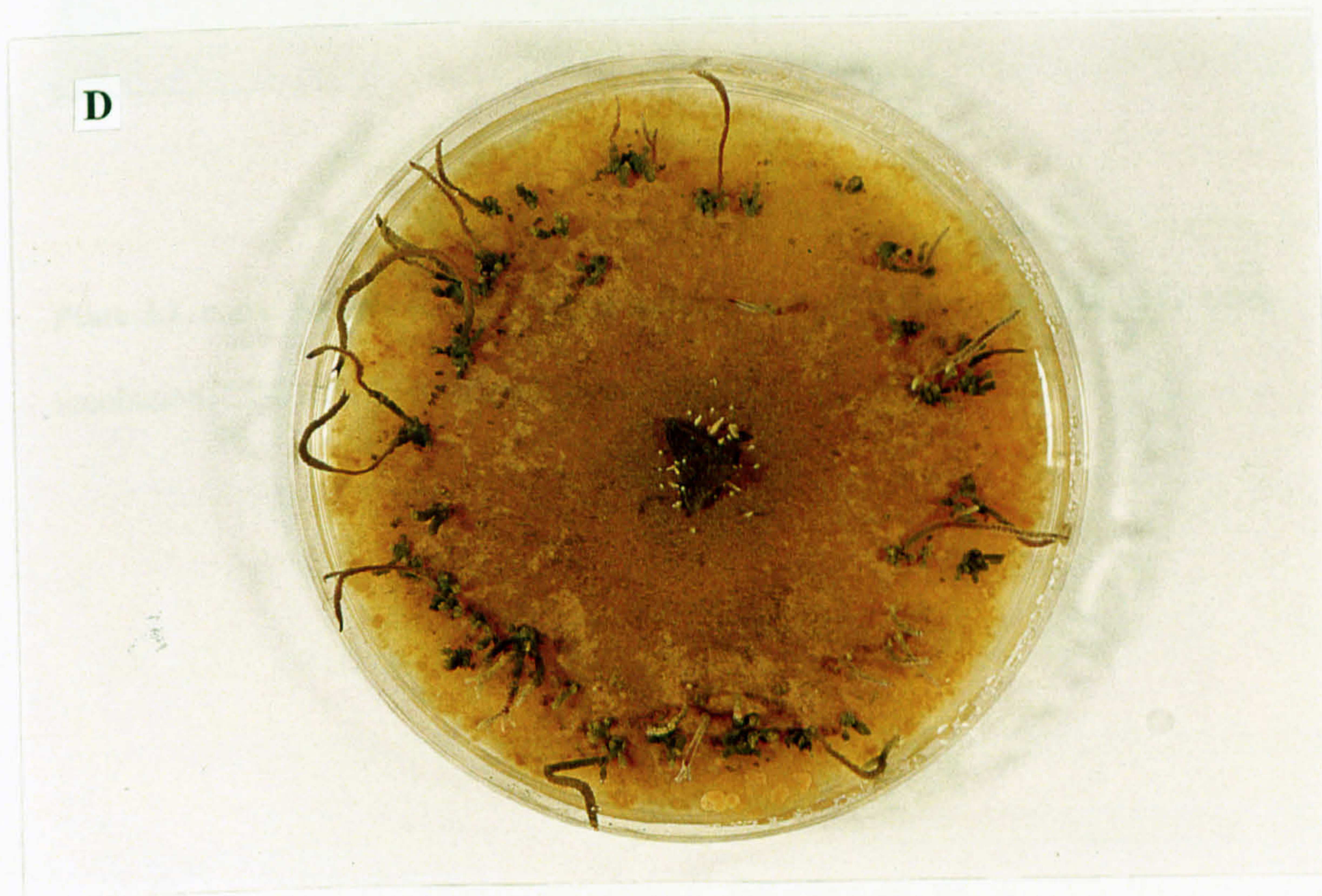
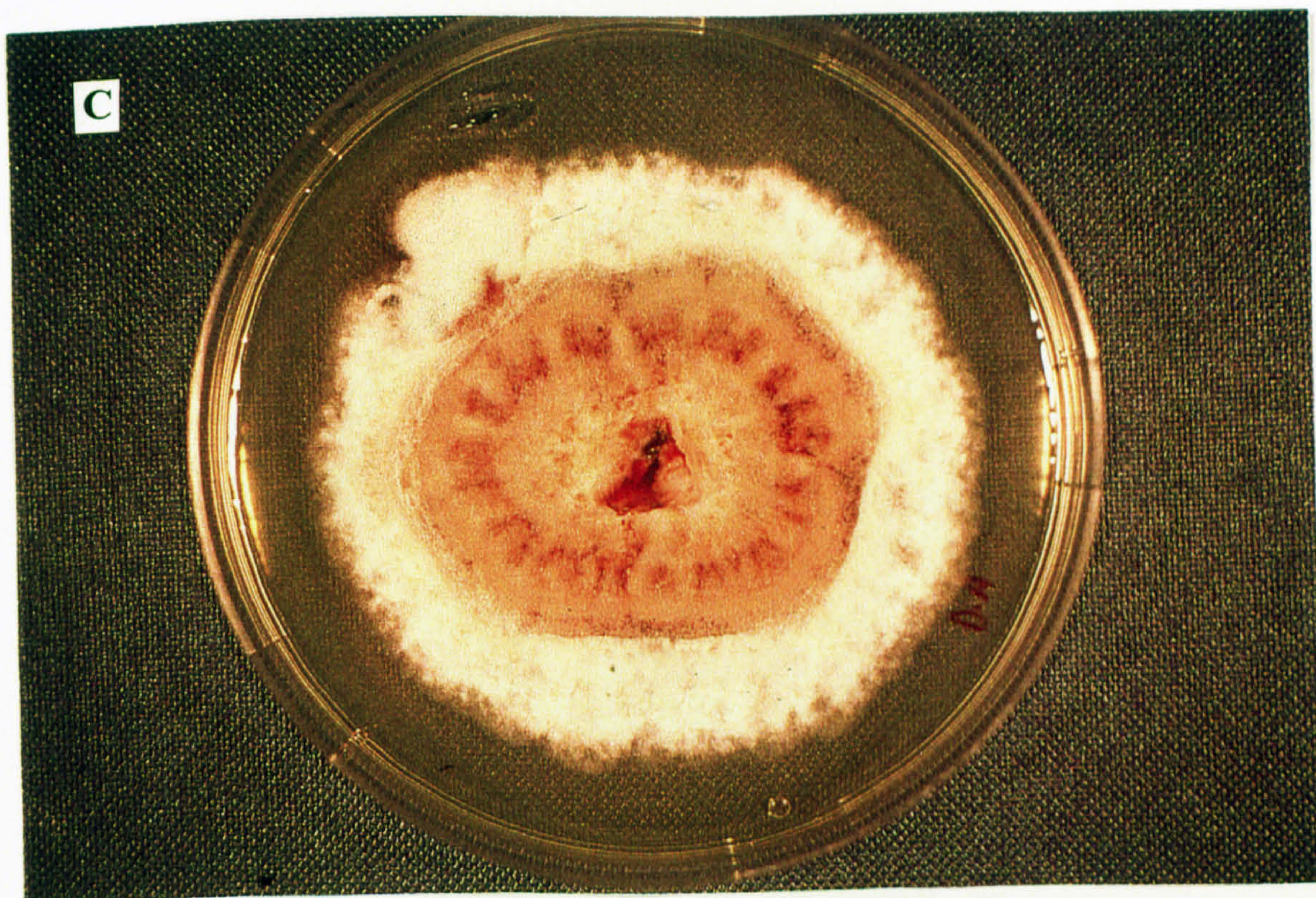


Plate 3.1. cont. Morphology of fungal isolates on malt extract agar (MEA) (2 week incubation, 25°C). C. *Drechslera dematioidea*. D. *Sarophorum palmicola*.



Plate 3.1. cont. Morphology of fungal isolates on malt extract agar (MEA) (2 week incubation, 25°C). *E. Epicoccum nigrum*.

3.2. *IN VITRO* GROWTH AND SECONDARY METABOLITE STUDIES WITH A *PHOMA* SPECIES USING GRAIN AND SEED EXTRACT AGARS.

Experiments were performed with a *Phoma* species using grain and seed extract media and malt extract agar (MEA) over a range of a_w levels and temperatures. These extract media represented simplified versions of substrates that could be developed subsequently for SSF work. The experiments were designed to investigate the influence of nutrient status, temperature and a_w and their interactions on growth and production of squalenstatin S1 by the *Phoma* species.

3.2.1. Stock cultures.

Stock cultures of *Phoma* sp. (IMI 332962) were stored at ambient temperature in 1.8 ml sterile water in 2 ml cryogenic vials (Nalgene, Merck, Poole, Dorset. UK.). Working cultures were prepared on malt extract agar plates (MEA) (Lab-M, Toddington, Bedfordshire. UK.) incubated at 25°C for 14 days.

3.2.2. Media preparation.

Starchy grain extracts were prepared from whole wheat and oat grain (see Section 3.3.1.). For both grain types 30g of grain were chopped in a Waring blender for 1 minute on the highest setting. The resulting material was added to 400 ml reverse osmosis (RO) water and autoclaved for 1 hour at 121°C. The extract was allowed to cool, then filtered through two thicknesses of muslin and made up to a volume of 500

ml with RO water. This suspension was used at 400 ml l⁻¹ in the final medium, equivalent to 24g l⁻¹ of original grain.

Oil seed rape extract (OSRE) was prepared by sonicating aliquots of 7.5g (1%) crude rapeseed oil (Cargill Ltd. Hull, North Humberside. UK.) into 750 ml RO water to produce a stable emulsion. A Soniprep instrument (Fisher, Loughborough, Leicestershire. UK) was set to maximum power and optimal frequency and the probe (standard type) was positioned so that the tip only was immersed in the water. The oil was run carefully down the shaft to produce a stable emulsion.

Malt extract medium (ME) was prepared using 30g l⁻¹ ME (Difco, East Molesey, Surrey. UK.) and 5g l⁻¹ mycological peptone (Lab-M.).

Agars were prepared from these extracts (Lab-M agar No. 2) at water activity (a_w) levels of 0.997, 0.995, 0.990, 0.980 and 0.960 by addition of appropriate weights of the non-ionic solute glycerol prior to autoclaving (Table 3.1.). Glycerol was used in this study since NaCl (ionic solute) has previously been shown to have an inhibitory effect on squalenstatin production (Baxter *et al.*, 1998). For each glycerol addition, an adjustment was made to the amount of grain or oil extract added to the medium to prevent a dilution effect occurring with lowered a_w (Table 3.2.). Similarly, adjustments were made to the concentrations of constituents used in the preparation of ME medium.

Table 3.1. Water activities of glycerol solutions at 25°C (Dallyn and Fox, 1980).

WATER ACTIVITY (a_w)	GLYCEROL ADDITION g. 100 ml H ₂ O
0.995	2.76
0.991	4.61
0.982	9.20
0.971	14.72
0.964	18.40

Table 3.2. Preparation of grain extract media over a range of a_w levels.

a_w	GLYCEROL (g)	WATER (ml)	EXTRACT (ml)	FINAL VOL. (ml)
0.998	0.0	450.0	300.0	750.0
0.995	20.7	441.7	308.3	770.7
0.990	34.6	436.2	313.8	784.6
0.980	69.0	422.4	327.6	819.0
0.960	138.0	394.8	355.2	888.0

All preparations were autoclaved at 121°C for 15 min and allowed to cool to approx. 50°C. Plates were then poured with each containing approx. 25 ml medium. Plate pouring was carried out in a laminar flow cabinet. The agar a_w was measured using a Humidat IC-2 instrument after calibration with standard salt solutions. Agar a_w was found to be at least within 0.005 of the required levels, and typically within 0.002.

3.2.3. Culture and growth conditions.

All agar plates were inoculated centrally with one 4 mm diameter plug of *Phoma* sp. taken from the margin of growing cultures. Agar plates were divided into three groups and incubated at 15, 20 and 25°C for a total duration of 30 days. For each experimental treatment (agar type, a_w , temperature) 10 replicate plates were incubated. Colony diameters were measured in two directions every 2-4 days. From this data mean colony radial growth rates (mm day^{-1}) were calculated.

3.2.4. Secondary metabolite analysis.

The methodologies of Glaxo Wellcome R & D. were employed for extraction and quantification of squalestatin S1. Three replicate plates from each treatment were destructively sampled at 10, 20 and 30 days. In each case 4mm diameter plugs were removed from the colony surface in a pattern of equal spacing. Colonies greater than 25mm diameter had 16 plugs removed. Colonies of 15-25 mm diameter had 7-8 plugs removed. Colonies of less than 15 mm diameter were not sampled.

Plugs were placed in 2 ml Eppendorf tubes (Sartorius, Epsom, Surrey. UK.) and stored, where necessary at -20°C. Metabolite extraction was carried out using an extraction solvent comprising 80% v/v acetonitrile (Aldrich, Gillingham, Dorset. UK.) and 0.03% v/v H_2SO_4 (BDH, Poole, Dorset. UK.) in water (Aldrich). For 16 plugs 1ml was added, for 8 plugs 0.5 ml was added and for 7 plugs 0.438 ml was added. The samples were incubated at 25°C for 1 hour with shaking every 15 minutes. The liquid was decanted and filtered through a 0.45 μm nylon syringe filter (Phenomenex,

Macclesfield, Cheshire. UK.). Filtered supernatants were transferred to 2 ml HPLC vials (Anachem, Luton, Bedfordshire. UK.)

Squalestatin S1 was quantified using a Gilson 715 HPLC system (Gilson, Villiers Le Bel, France). Aliquots of 10 µl were injected automatically using a Gilson 231 XL sampling injector. The column used was a 5 µm C6 spherisorb 150 x 4.6 mm. type (Phenomenex) with a guard column of the same material. The mobile phase was 55% v/v acetonitrile and 0.015 % v/v H₂SO₄ in water. Running conditions were 1 ml min⁻¹ flow rate, total run time 20 minutes, and UV detection at 210 nm. Extracts were analysed against calibration curves of S1 over the range 5 - 50 µg ml⁻¹ (see Appendix 1). From the HPLC results mean yields of S1 were calculated, expressed as µg g⁻¹ wet weight agar (see Section 3.11.3.).

3.2.5. Extraction efficiency.

Analytical losses were accounted for by determining extraction efficiency. Extraction efficiency from the agar was determined experimentally by two methods :

1. A 2 ml sample of 200 µg ml⁻¹ S1 was added to 18 ml molten wheat extract agar (WEA) and MEA. After the media had set 4 mm diam. plug samples were taken and analysed as described in Section 3.2.4.
2. Agar plugs (4 mm diam.) for all media types were treated with extraction solvent containing 50 µg ml⁻¹ S1 then extracted and analysed as described in Section 3.2.4.

For extraction efficiency results see Appendix 1.

For both methods, “unspiked” plugs were run alongside the treated samples to act as controls. The mean weight of 4 mm agar plugs, necessary for calculating extraction efficiencies, was determined for MEA, WEA and oil seed rape extract agar (OSREA) by weighing 5 replicate plugs of each type. From this a mean weight for each agar type and mean weight for all types was obtained.

3.2.6. Calibration curves.

A stock solution of 100 $\mu\text{g ml}^{-1}$ S1 was prepared in extraction solvent. From this a range of concentrations were prepared (10, 20, 40, 50 $\mu\text{g ml}^{-1}$) and stored frozen at -80°C prior to use. Aliquots of each concentration were used at the beginning of each HPLC run to construct a calibration curve. The correlation coefficient for these plots was typically > 0.99.

3.3. INITIAL WORK WITH SOLID SUBSTRATES.

The first phase of work involved selecting a range of solid substrates to test in solid substrate fermentation systems. A similar range of materials as used in extract work with *Phoma* sp. were used, together with various mixtures and processed materials.

3.3.1. Solid substrates used in the study.

The initial aim was to obtain a number of materials that demonstrated suitable properties in terms of:

1. Nutrient content.
2. Particle size.
3. Response to wetting.
4. Response to autoclaving.

The most obvious choice was heterogeneous agricultural materials of various types since these are readily available at consistent quality and have been used extensively in the past as SSF media. Most of the substrates used were based on cereals, which are the fruits of cultivated grasses (family *Gramineae*). The mature grain of all common cereals consists of carbohydrates (approx. 80%), proteins, lipids, mineral salts and small quantities of vitamins and other trace substances (Kent, 1978). On a nutritional basis they represent good substrates for fungal growth, as is demonstrated by the rapid spoilage of poorly stored grain, a serious economic problem world-wide.

The following substrates were utilised in experimental work:

1. *Whole wheat grain (Triticum spp.)* (R. D. Hawkings, Stagsden, Beds.).

In wheat the lemma and palea (outer coats) readily separate from the grain during threshing (forming the chaff). Wheat is therefore described as a “naked” caryopsis (grain) (Kent, 1978). Average particle size is 6x3mm.

2. Whole oat grain (*Avena* spp.) (R. D. Hawkings, Stagsden, Beds.).

The oat grain incorporates a fused glume (the palea and lemma) which constitutes the husk. The grain is therefore described as a coated caryopsis. In this study the grain was used with the husk intact. The carbohydrate content of oats (typically 79%) is slightly lower than for wheat. The fat content is relatively high (typically 5%, approx. twice that of wheat). Average particle size is 11x3mm.

3. Oil seed rape (rapeseed) (*Brassica napus*) (R. D. Hawkings, Stagsden, Beds.).

Oil seed rape is not a cereal, but is a member of the cabbage family (*Cruciferae*). The seeds are small (average 2mm diameter) and extremely uniform. Oil seed rape has a high oil content. This substrate is therefore fundamentally different from the others, being “oily” rather than “starchy”.

4. Spanish maize (*Zea mays*) (University of Lleida, Lleida, Spain).

Maize grains are naked caryopses. However the pericarp and testa are fused to form a “hull” which corresponds to, but is anatomically distinct from, the husk in other cereals. The grains are larger than those of other cereals, in this case an average 10 x 9 mm.

5. Soya beans (*Glycine max*) (Bedford Wholefoods Ltd., Bedford, UK.).

Soya is not a cereal, it is a legume with a very high protein content, and is therefore fundamentally different from the other materials selected as candidate substrates in this work. Average bean size is 7mm. diameter.

6. *American long grain rice (Oryza sativa)* (Tilda, Rainham, Essex UK.).

Rice is morphologically similar to oats, the grains being coated caryopses. However, in this study a commercially available “polished” rice was used with the husk removed. Average grain size is 7 x 2 mm.

7. *Shredded wheat* (Nabisco Ltd.).

Shredded wheat is a “ready to eat” breakfast cereal produced from processed wheat. “White” (starchy) wheat is used for shredding. The whole grain is cleaned and cooked by steaming until it is soft and “rubbery” (starch gelatinised). The grain is cooled, then fed to shredders forming a mat. The mat is cut into tablets and baked at 260°C for 20 min. The product is then dried to 1% moisture content, cooled and packaged (Kent, 1978).

8. *“Weetabix”* (Cereal Partners Ltd, Welwyn Garden City, Hertfordshire, UK.).

“Weetabix” is a processed wheat breakfast cereal product similar in usage to shredded wheat.

9. *“Shreddies”* (Cereal Partners Ltd, Welwyn Garden City, Hertfordshire, UK.).

“Shreddies” are another breakfast cereal product based on wheat. This product is probably manufactured in a similar way to shredded wheat.

10. *Wheatflakes* (Bedford Wholefoods Ltd., Bedford UK.).

Wheatflakes are produced from good quality unsprouted wheat. Cleaned, de-stoned grain is micronised (toasted) and rolled to form a flake. Average flake size is 8 x 9 mm.

11. *Bulgar wheat* (Bedford Wholefoods Ltd., Bedford, UK.).

Bulgar is one of the oldest cereal-based foods, and has been consumed for centuries, particularly in the countries of the Middle East. It can be made from most wheat types, but a hard wheat such as durum is normally preferred. The grain is cooked in a multistage process where the moisture level and temperature are gradually raised until the water content is 40%. The grain is then steamed under pressure until the starch content is partially gelatinised. Finally the product is dried and pearled or cracked. Bulgar is used in a similar way to rice and has been supplied, as a rice substitute, to the Far East as part of the programme of American aid to famine areas (Kent, 1978). Coarse, fine and very fine bulgar is available. The average particle size of the material used in this study is 3 x 3 mm.

12. *Couscous* (Bedford Wholefoods Ltd., Bedford, UK.).

Although superficially resembling bulgar, couscous is actually a "paste" product (similar to pasta) made from durum wheat semolina (a coarse, "gritty" flour). Couscous is one of the staple foods of the countries of North Africa (Dick & Matsuo, 1988). Average particle size is 2 mm. diameter.

13. *Flaked millet (Pennisetum typhoides)* (Squirrels Health Foods, Burnham, Berks. U.K.).

Pearl millet is a staple human food in Asia and Africa. It is a small starchy grain with a relatively high protein and fat content (Rooney & McDonough, 1987). Flaked millet is produced by rolling pearl millet, presumably in a similar process to the production of flaked wheat. Average size of flakes is 3mm x 3mm. In this study, flaked millet was used as a second nutrient phase in some experiments.

14. *Perlite* (Pioneer Supamix Ltd., Nuneaton, Warwickshire. UK.).

Perlite is a natural inert mineral product normally used in horticulture as a soil improver. It was used in this study as a nutrient free second phase in some experiments. Average particle size is 4 x 3 mm. However, the particle size of this material is highly variable with a significant proportion of dust.

15. *TerraGen medium A* (Terragen Discovery Ltd., Slough, Berkshire. UK.)

Medium A is a relatively complex multi-component medium used routinely by TerraGen in SSF work.

Materials (1)-(4) were stored at nominally 4°C in sealed containers in darkness prior to use. Other substrates were stored at ambient temperature in darkness. The main nutritional components for the substrate types are shown in Table 3.3.

Table 3.3. Nutritional composition of substrates.

Substrate	Protein %	Fat %	Carbohydrate %	Minerals %
Wheat ¹	10.5	2.6	78.6	1.8
Oat (whole) ¹	11.6	5.2	69.8	2.9
Rapeseed ²	22.4	45.9	NA	NA
Spanish maize ³	7.5	3.5	74.0	NA
Soya bean ⁴	39.0	20.0	16.0	NA
American rice ⁵	7.7	0.6	79.6	0.6
Wheat flakes ⁶	13.4	2.3	67.6	NA
Bulgur wheat ⁶	9.7	1.7	76.3	NA
Couscous ⁶	13.5	1.9	72.5	NA
Shredded wheat ¹	11.3	2.3	74.0	NA
Weetabix ¹	10.9	1.9	77.0	NA
Flaked millet ⁷	12.1	5.0	69.4	NA

Sources of data: ¹ Kent (1978). ² American Oil Chemists Society. ³ University of Lleida, Spain. ⁴ Holland & Barrett, Nuneaton. UK. ⁵ Tilda Rice, Rainham, Essex. UK. ⁶ Bedford Healthfoods Ltd. Bedford, UK. ⁷ Rooney & McDonough, (1986).

(NA – not available)

3.3.2. Moisture sorption isotherms.

Moisture sorption isotherms were prepared for all materials that appeared suitable for use as solid substrates after initial hydration. One mixture, wheat and millet flakes (50:50) was also included. For each type a series of 7 - 10 hydration levels were prepared to give a moisture range of approx. 15 - 40% wet weight water. Aliquots of approximately 2.5 - 10g substrate (depending on type) were placed in Universal bottles and the appropriate amount of reverse osmosis (RO) water was added. A typical hydration scheme is shown in Table 3.4. For substrates 1-3 two replicates were prepared at each treatment level. For the remaining substrates, three replicate treatments were employed. After water addition the bottles were allowed to equilibrate overnight and were then autoclaved (15 min at 121°C). The Universal bottles were then stored for four days at 5°C with daily shaking to allow for water to re-equilibrate fully with the substrate. At this stage moisture contents were determined by drying weighed samples overnight (15 hours) at 106 °C then re-weighing. Water activity (a_w) measurements were made using a Humidat IC II electronic hygrometer (Novasina, Pfaffikon, Switzerland) in a controlled temperature room at 25°C, allowing one hour equilibration before readings were taken. The Humidat instrument was calibrated immediately before use in the range 75.3 - 98.0 equilibrium relative humidity (ERH) using standard salt solutions. For each substrate type, isotherms were constructed by plotting the percentage moisture content against a_w . In some instances, added water against a_w was also plotted.

Table 3.4. Typical hydration scheme of substrates for preparation of isotherms.

Treatment	Water addition ml 10g ⁻¹ grain
1	0.0
2	0.25
3	0.5
4	1.0
5	1.5
6	2.0
7	2.5
8	3.0
9	3.3
10	4.0

3.4. SMALL SCALE METABOLITE STUDIES WITH SOLID SUBSTRATES.

Experiments were performed using the fungal spp. detailed in Section 3.1.2. and the solid substrates detailed in Section 3.3.1. to investigate the ability of the organisms to produce secondary metabolites with these various media over a range of a_w levels. It was intended that this information would then be used to select a suitable “model system” (organism/substrate combination) to carry out further work.

3.4.1. Substrate preparation.

For each substrate type a series of samples was prepared to give a range of a_w levels for experimental work. The a_w levels employed were: 0.998 (i.e. saturated), 0.990, 0.980 and 0.970/0.960. These a_w levels were set with reference to the moisture sorption isotherms detailed in Chapter 4.2. The isotherms were used to obtain the appropriate percentage water contents corresponding to the required a_w levels and the necessary amounts of RO water were added to weighed aliquots of each of the substrates. For the inert medium (perlite) it was not possible to produce an isotherm (see Section 5.2.) so the appropriate a_w levels were set using glycerol solutions (see Table 3.1.).

3.4.2. Experimental treatments.

Experimental treatments were of three main types:

1. “Two phase” treatments: In these experiments, the substrates were added in a “two phase” arrangement with each jar containing three parts of a nutrient substrate and one part of the inert substrate. For these treatments the nutrient medium was added first then a spatula was used to “sweep” a quadrant equal to one quarter of the base

area of the jar. The space was then filled to the same height with perlite. The inert second phase was included in these treatments since there is evidence that this arrangement can stimulate secondary metabolite production in some systems. For all of these experiments water used for wetting the substrates was supplemented with nutrient additions. The supplement addition consisted of 0.1% mycological peptone and 0.1% malt extract except for the inert medium (perlite) where 0.1% mycological peptone alone was used (all supplements supplied by Lab-M). In other experiments a two-phase nutrient system was employed (e.g. couscous + millet).

2. Simple “single phase” treatments: In these experiments a simple single nutrient phase was employed without an inert phase or supplements.
3. Comparative treatments: A series of experiments were performed (using wheat flakes), to compare directly the effects of using (i) the inert second phase (perlite), (ii) a nutrient second phase (millet), (iii) the trace nutrient supplement and (iv) wheat flakes alone. Results were used to assess the significance of these treatment types on secondary metabolite production.

3.4.3. Treatment preparation.

For each a_w level a single bulk sample of each substrate was prepared sufficient for experimental needs. This was autoclaved once (15 min. at 121°C) and the substrate was dispensed into sterile treatment jars and autoclaved again for the same duration.

The two autoclaving treatments were carried out over subsequent days. After the second autoclaving treatment the jars were allowed to cool and re-equilibrate for 24 hrs before they were used in experimental work. Jars used for individual treatments were 72 mm plant tissue culture vessels with polypropylene microporous vented lids (Sigma, Poole, Dorset. UK.). All jars were filled to a constant volume of approx. 40cm³ (substrate depth 15 mm). Each treatment was prepared in triplicate.

3.4.4. Inocula preparation and inoculation of jars.

For details of the fungal spp. used in individual experiments see Table 3.5. To prepare inocula, all isolates were grown on MEA plates at 25°C for two weeks. Plates were harvested by addition of 10 ml of a sterile mineral salts medium (BS 2011, British Standards Institute, Milton Keynes, Bucks.) and gentle scraping with a sterile loop to release spores and/or hyphal fragments. This material was then filtered through glass wool and the filtrate used immediately as the inoculum. In the case of *S. palmicola*, which sporulated freely in these conditions, the spore inoculum was added to the treatment jars using an air brush (Badger, UK.). The airbrush was calibrated to deliver 0.5 ml in 2 secs. and this volume was then sprayed uniformly into each jar. In the case of the other three organisms, which did not produce spores, a hyphal fragment inoculum was prepared in the same way as the spore inoculum and the jars were inoculated with 0.5 ml of filtrate by pipette. Harvesting of fungal cultures and inoculation of jars was carried out in a class 2 laminar flow cabinet (MDH Ltd., Andover, Hants. UK.).

Table 3.5. Scheme of solid substrate experiments.

Substrate	Organism	Inert phase	Supplement
TerraGen medium A	All	✓	✓
Whole wheat	All	✓	✓
Rapeseed	All	✓	✓
Whole oats	All	✓	✓
Maize	<i>S. palmicola</i>	✓	✓
	<i>C. cassiicola</i>		
Rice	<i>S. palmicola</i>	✓	✓
	<i>C. cassiicola</i>		
Soya beans	<i>S. palmicola</i>	✓	✓
	<i>C. cassiicola</i>		
Wheat flakes	<i>E. nigrum</i>	✓	✗
Wheat flakes	<i>E. nigrum</i>	✗	✓
Wheat flakes	<i>E. nigrum</i>	✗	✗
Wheat flakes + millet (50:50)	<i>E. nigrum</i>	✗	✗
Bulgar wheat	<i>E. nigrum</i>	✗	✗
Couscous	<i>E. nigrum</i>	✗	✗
Shredded wheat	<i>E. nigrum</i>	✗	✗
Couscous + millet (50:50)	<i>E. nigrum</i>	✗	✗

3.4.5. Control samples.

For each solid substrate type uninoculated control samples (3 replicates) were incubated with the other samples and processed in the same way to enable substrate components to be eliminated from secondary metabolite profiles.

3.4.6. Incubation of jars.

Inoculated treatments were placed in transparent sealed boxes such that jars of the same a_w value were incubated together. The humidity of the box interiors was controlled by the addition of glycerol solutions (set to the appropriate a_w level), in open containers. These solutions were changed once each week. The boxes were placed in a temperature controlled room at $25\pm 2^\circ\text{C}$ for one week then $18\pm 2^\circ\text{C}$ for a second week (total experiment duration two weeks). The boxes were illuminated diurnally using a UV lamp (Interpet Ltd., UK.). Growing cultures were inspected regularly for any signs of contamination. At the end of the incubation period a growth assessment was attempted, in terms of:

1. Mycelium - % cover, diffuse or dense (diffuse = underlying substrate visible, dense = underlying substrate obscured).
2. Sporulation – present / absent (% cover if appropriate).
3. Pigmentation – present / absent, distribution.

3.4.7. Secondary metabolite extraction.

At the end of the incubation period each jar was prepared for solvent extraction by coarse chopping using a sterile knife or spatula followed by freeze-drying of the entire jar contents to eliminate water (Edwards high vacuum freeze drier unit, Edwards, Crawley, Sussex. UK.) The freeze-dried material was then dry “macerated” using a food processor (Kenwood, Havant, Hants. UK.) for approx. 30 sec on the “high” setting. Alternatively, for material which was already well divided, the jar contents were briefly ground manually with a pestle. 50 ml of HPLC grade methanol (Prolabo, Fontenay, France.) was then added to each jar and all jars were placed in a fixed frequency ultrasonication bath (Decon Ultrasonics, Hove, Sussex. UK.) for 30 min to optimise solvent extraction. In initial studies the entire methanol content of each jar was filtered and recovered using a custom designed vacuum filtration rig (TerraGen design), but in later studies a small volume of extract was collected in a 2 or 5 ml syringe and filtered through a 0.45µm syringe filter (Sartorius, Epsom, Surrey, UK.). Filtrate samples were placed in HPLC vials (Chromacol, Welwyn Garden City, Herts. UK.) and analysed immediately or placed in a freezer at –20°C pending analysis.

3.4.8. HPLC analysis.

Samples were analysed by HPLC using a Waters Alliance 2960 system with a gradient method and photodiode array detection (Waters 996) (Waters, Millipore, Milford, MA. USA.), Waters symmetry C8 column, and dedicated Waters

“millennium” software. (Other chromatography details confidential to TerraGen Discovery Ltd.).

3.4.9. Assessment of secondary metabolites.

All fungal species used in this study had previously characterised metabolite profiles on medium A (work carried out at TerraGen). For each fungus a number of target metabolites had been selected from these profiles for particular attention in this project. The metabolites were selected on the basis of:

1. Novelty, with reference to TerraGen in-house and outside databases consisting of libraries of mass spectrometer fragments and UV spectra.
2. Levels produced in initial studies previously conducted by TerraGen. Minor peaks and metabolites that could be transient in nature were not selected.

The objective of this part of the study was to construct metabolite profiles for the present range of solid substrates and compare these with that obtained for medium A, and to select a suitable fungus/ substrate to use as a model system for further work. It was not within the scope of this project to differentiate between primary and secondary metabolites produced in these experiments, although the specific target metabolites previously selected were all secondary metabolites. In addition, the later eluting metabolites (typically beyond 9 min), which were more likely to be fatty acids and other products of primary metabolism, were omitted from some profiles. Specific

details are given in relevant sections. To allow a direct comparison medium A was itself included in this series of experiments. However, it could not be used extensively in the project as a whole since its formulation is commercially sensitive. The key criteria for selection of a suitable fungus/substrate system were (i) a metabolite profile showing similar or increased titres to those obtained for medium A, (ii) good representation of target metabolites and perhaps (iii) new metabolites not previously seen (particularly at lower a_w levels).

3.5. INVESTIGATION OF TEMPORAL DEVELOPMENT OF METABOLITES.

Previous small-scale studies in this project considered secondary metabolite development at one time point only (i.e. 2 weeks). This experiment was carried out to investigate the temporal changes in metabolite profiles and titres over a range of a_w levels produced by *E. nigrum* cultured on bulgar wheat.

3.5.1. Preparation of experimental treatments.

Bulgar wheat was prepared at a_w levels of 0.998, 0.990, 0.980 and 0.970.

Experiments were carried out in tissue culture jars as described in Section 3.4.3.

For each a_w treatment sufficient jars were prepared to destructively sample in triplicate at days 0, 4, 7, 10, 14, 18 and 21.

Treatment preparation, solvent extraction and secondary metabolite analysis were carried out as described in Sections 3.4.3., 3.4.7. and 3.4.8. respectively. Incubation

temperature differed from previous studies in that there was no step-down in temperature from 25°C to 18°C after one week. This was because it was intended to follow metabolite development under constant conditions. Other incubation conditions were as described in Section 3.4.6.

3.5.2. Agitated treatments.

An additional set of jars was prepared at 0.998 a_w and incubated in a reciprocating shaker (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey. UK.) set to 0.5 cycles sec^{-1} (other incubation conditions unchanged) to investigate the effects of continuous shaking on secondary metabolite development.

3.5.3. Investigation of changes in a_w level.

In addition to analysis for secondary metabolites, a_w was measured for all treatments at the time of sampling (method as described in Section 3.3.2.). This procedure was carried out to follow any changes in a_w that might occur in the course of fungal colonisation of the substrate.

3.5.4. Preparation of inoculum.

In these experiments the inoculum used was in the form of pre-inoculated bulgar wheat. This was prepared by adding 100ml RO water to 100g bulgar wheat in a 200ml screw cap bottle, to give an a_w level of approx. 0.990. This was autoclaved twice over consecutive days then inoculated with ten 4mm cork borer plugs taken from the margin of a MEA plate culture of *E. nigrum* (plate cultured for one week at 25°C). The inoculated bulgar wheat was incubated for ten days at 25°C then used directly to inoculate experimental treatments at a level of approx. 1.5g per treatment (equivalent

to approx. 6.5% substrate addition). Preparation of the inoculum and dispensing into jars was carried out in a class 2 laminar flow cabinet.

3.6. INVESTIGATION OF THE EFFECTS OF AERATION AND AGITATION AT SMALL SCALE USING *E. NIGRUM* GROWING ON BULGAR WHEAT.

This study was carried out to investigate the influence of continuous forced aeration on growth and secondary metabolite production of *E. nigrum* growing at small scale on bulgar wheat. This treatment was compared directly with intermittent agitation (as another form of aeration) and with normal static incubation.

3.6.1. Experimental treatments

All experimental treatments were set up in 72 mm plant tissue culture jars. Bulgar wheat was set to a_w 0.990. Preparation of jars, inoculation, incubation and analysis for secondary metabolites were otherwise as described in Sections 3.5.1. and 3.5.4.

1. Aerated jars.

Aerated treatments were set-up as shown in Fig. 3.1. Aeration was via humidified filter sterilised compressed air set to a flow rate of 0.75 litre min⁻¹ using a rotameter (Platon Ltd., Basingstoke, Hants. UK.). Filter sterilisation of compressed air was via an in-line HEPA 0.3µm glass microfibre filter (Whatman, Arbor Technologies Inc., Ann Arbor, MI. USA.).

Filter-sterilised compressed air was humidified by bubbling through a sterile glycerol solution at a p_{H_2O} of 0.990. The glycerol solution was housed in a 3 litre medical flask sealed except for air entry and exit ports, and autoclaved prior to use. Humidification of air was carried out to prevent desiccation of jar contents. The p_{H_2O} of the humidification

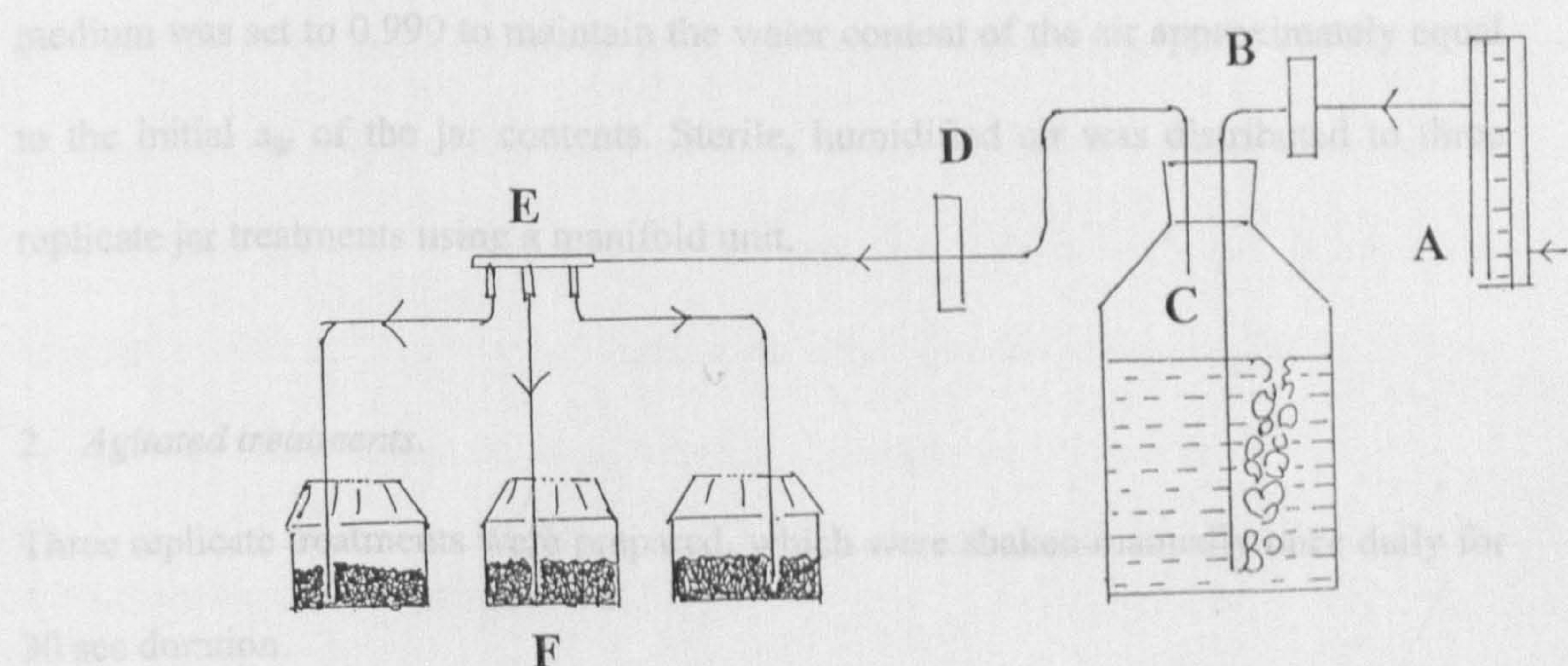


Figure 3.1. Schematic diagram of aerated system for *E. nigrum* growing on bulgar wheat in small scale jars. A – rotameter, B, D – air filters, C – humidifier bottle, E – air distribution manifold, F – treatment jars.

Filter-sterilised compressed air was humidified by bubbling through a sterile glycerol solution at a_w 0.990. The glycerol solution was housed in a 3 litre conical flask sealed except for air entry and exit ports, and autoclaved prior to use. Humidification of air was carried out to prevent desiccation of jar contents. The a_w of the humidification medium was set to 0.990 to maintain the water content of the air approximately equal to the initial a_w of the jar contents. Sterile, humidified air was distributed to three replicate jar treatments using a manifold unit.

2. *Agitated treatments.*

Three replicate treatments were prepared, which were shaken manually once daily for 30 sec duration.

3. *Static treatments.*

Three replicate treatments were prepared, which were incubated statically.

3.7. FIRST SOLID SUBSTRATE SCALE-UP STUDY.

In this study growth and secondary metabolite development were followed for *E. nigrum* growing on bulgar wheat at a ten-fold increase in substrate volume. This was carried out to investigate metabolite production at a first level of scale-up, and to identify any problems associated with larger substrate volumes.

3.7.1. Preparation of experimental treatments.

Experimental treatments were prepared in 500cm³ Duran bottles (Schott, Fischer Scientific Ltd., Loughborough, Leicestershire, UK.) at a_w levels of 0.998, 0.990, 0.980 and 0.970. Each treatment bottle was prepared separately by addition of 150g of dry bulgar wheat followed by the appropriate addition of RO water with reference to the moisture sorption isotherm constructed for bulgar wheat (Fig. 4.8). Bottles were stoppered using polyurethane foam bungs to allow gas exchange during incubation. Bottles were allowed to equilibrate, then the final substrate volume in each was adjusted to 400cm³. For “wetter” treatments, this required the removal of up to 100g of material. Treatments at 0.970 a_w required no adjustment of volume.

3.7.2. Inoculation and incubation.

Inoculation was as described in Section 3.5.4., at a level of 3-4% of substrate volume. Each treatment level was prepared in triplicate with two separate groups, one incubated statically and one incubated with intermittent manual shaking (once daily, 30 sec duration). Bottles were incubated at $25 \pm 2^\circ\text{C}$ for 14 days in a lidded glass tank with the internal atmosphere set to approx. 0.970 using glycerol solutions (see Table 3.1.). Diurnal illumination was provided by UV lamps.

3.7.3. Sampling and secondary metabolite analysis.

At the end of the incubation period samples were taken from each bottle for secondary metabolite analysis. To allow direct comparison with previous small-scale studies, 40cm³ of material was taken from each bottle. Bottles were shaken vigorously to break up clumps, and the sample was taken in the form of a “core” through the

substrate depth. Secondary metabolite extraction and analysis were as described in Sections 3.4.7. and 3.4.8. respectively.

3.8. FIRST LABORATORY SCALE FERMENTER STUDY.

In this study a second level of scale-up was attempted, from 400cm³ wet substrate to 3000 cm³ (3 litre) wet substrate, an increase of x 7.5. This experiment involved the design of a simple fermenter apparatus which included provision for aeration and agitation of the substrate during incubation.

3.8.1. Fermenter design.

The fermenter design consisted of two coupled 5 litre Duran bottles (Schott, Fischer Scientific Ltd., Loughborough, Leicestershire. UK.), one acting as the fermenter and the other acting as the humidifier vessel. Both of the vessels were fitted with air entry and exit ports in the lids. The fermenter vessel was filled to a volume of 3 litre with bulgar wheat wetted and equilibrated to 0.990 a_w . The humidification vessel was charged with 3 litre of RO water. Pure water with the theoretical ability to fully saturate the air stream was chosen for this study since it was considered that water uptake would be below the theoretical maximum, and there were indications from the previous aerated study of drying effects. The vessels were autoclaved twice over subsequent days (121°C; 50 min) in an Astell Hearson AAJ040 portaclave (Astell Hearson Ltd., Catford, London. UK.). The extended autoclaving time was used in an effort to remove bacterial contamination. The fermentation apparatus arrangement was as shown in Fig. 3.2. Compressed air flow rate was set to 2 litre min⁻¹ using a rotameter

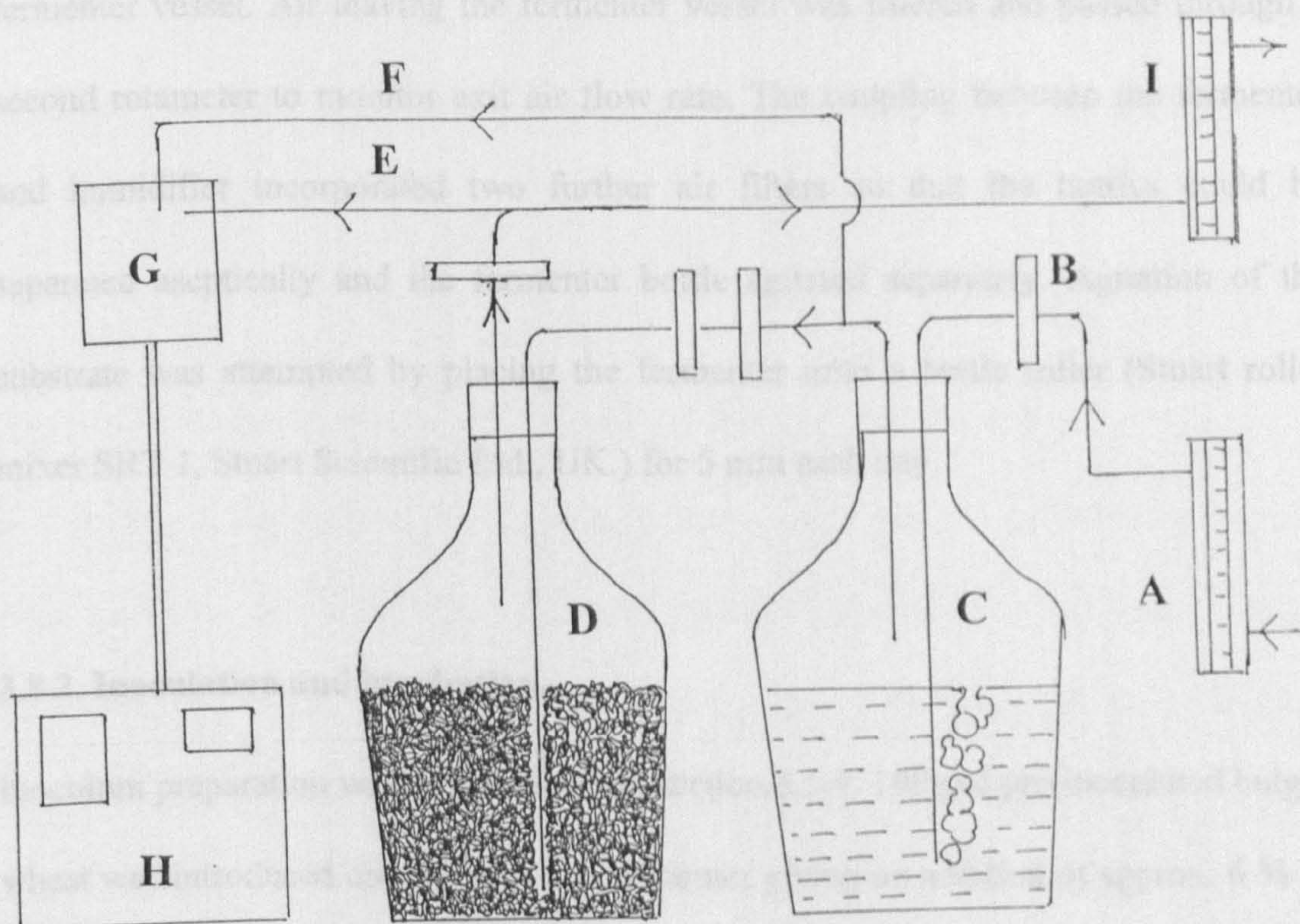


Figure 3.2. Schematic diagram of fermentation system using 5 litre Schott bottles. A – input air rotameter, B – air filter, C – humidifier bottle, D – fermenter bottle, E – output air a_w measurement line, F – input air a_w measurement line, G - a_w sensor, H – a_w meter, I – output rotameter.

and the air was passed through an air filter and humidifier before entering the fermenter vessel. Air leaving the fermenter vessel was filtered and passed through a second rotameter to monitor exit air flow rate. The coupling between the fermenter and humidifier incorporated two further air filters so that the bottles could be separated aseptically and the fermenter bottle agitated separately. Agitation of the substrate was attempted by placing the fermenter onto a bottle roller (Stuart roller mixer SRT 1, Stuart Scientific Ltd., UK.) for 5 min each day.

3.8.2. Inoculation and incubation.

Inoculum preparation was as described in Section 3.5.4. 100g of pre-inoculated bulgar wheat was introduced directly into the fermenter giving an addition of approx. 6 % of fermenter contents.

During the course of the experiment ambient and fermenter vessel wall temperatures were recorded daily together with exit and entry air flow rates. Measurement of entry and exit air a_w was facilitated by diverting air flow into a measuring box fitted with a Novasina SM114 a_w probe connected to a TH200 meter (Novasina, Pfaffikon, Switzerland). The fermenter apparatus was run for 14 days in a temperature controlled room at $25\pm 2^\circ\text{C}$ with diurnal UV lighting.

3.8.3. Sampling and secondary metabolite analysis.

At the end of the incubation period three replicate samples of 40 cm³, representing an overall average sample, were taken from the fermenter contents for secondary metabolite analysis. Extraction and analysis methods were as described in Sections 3.4.7. and 3.4.8.

3.9. SECOND LABORATORY SCALE FERMENTER STUDY.

In this study a laboratory scale submerged liquid fermenter was modified for use as a solid substrate fermenter. The substrate volume used in the study was 3 litres, equivalent to a x 7.5 increase compared to the first scale-up study. This fermenter design incorporated continuous aeration of the substrate, but no agitation capability was included.

3.9.1. Fermenter design.

A Bioengineering 2000 KLF 3 litre laboratory scale fermenter (Bioengineering AG, Wald, Switzerland) was utilised in this study. The fermenter essentially consisted of a glass cylindrical column (250 mm length, 117 mm internal diameter) with stainless steel plates top and bottom. The plates are drilled with a pattern of holes for fitting ancillary equipment, and bolt onto the glass column via ring and seal assemblies. The fermenter apparatus was set-up as shown in Fig 3.3 and Plate 3.2. The mechanical stirrer shaft and seal assembly were removed from the base plate and replaced with a rubber bung which was sealed into place using silicone rubber and positioned flush

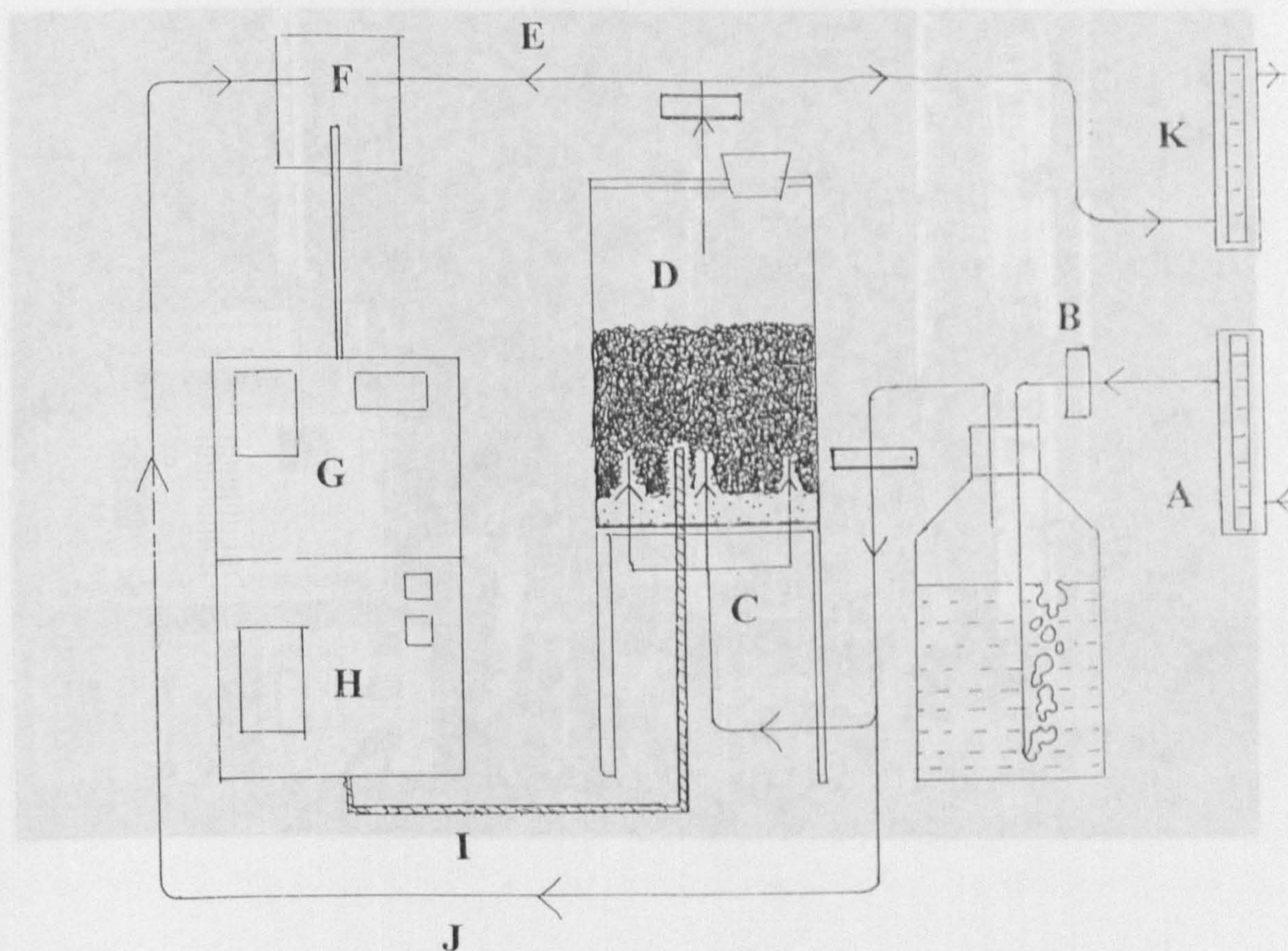


Figure 3.3. Schematic diagram of fermentation system incorporating laboratory scale submerged liquid fermenter. A – input air rotameter, B – air filter, C – air distribution manifold, D – fermenter, E – output air a_w measurement line, F - a_w sensor, G – a_w meter, H – temperature meter, I – temperature sensor, J – input air a_w measurement line, K - output rotameter.

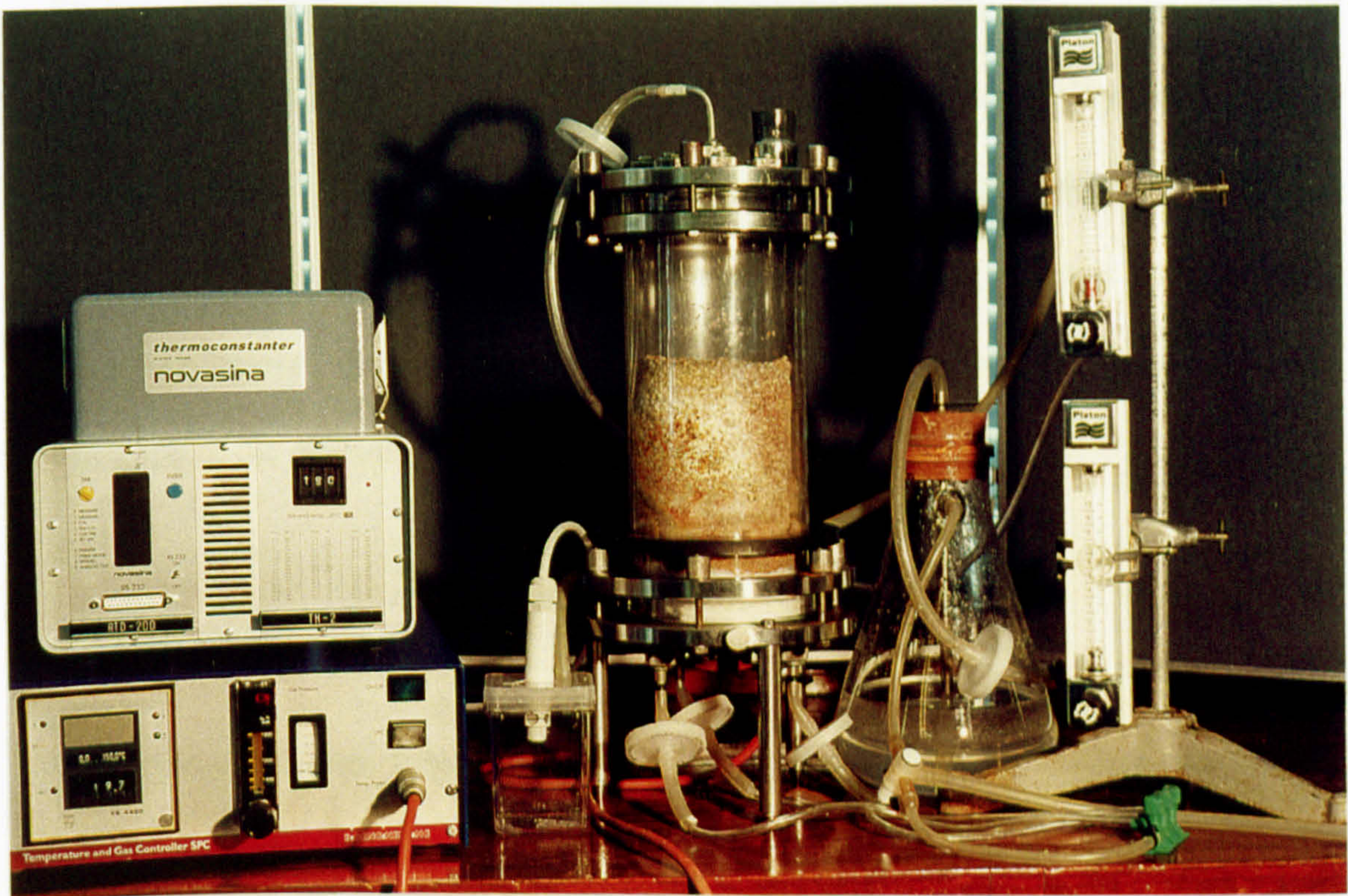


Plate 3.2. Fermentation apparatus incorporating a Bioengineering AG pilot scale submerged liquid fermenter. Ancillary monitoring equipment is shown at left.

with the fermenter floor. The base plate was then fitted with a temperature monitoring finger and three equidistant air entry ports using the standard needle and septum type fittings. A manifold was used to distribute the air to the entry ports. The remaining ports were sealed with blanking plugs. Similarly, the top plate was fitted with a single air exit port and the remaining holes blanked off. A larger hole, normally housing the dissolved O₂ sensor, was fitted with a removable rubber bung to act as an inoculating port. The base of the assembled fermenter was packed with glass wool to prevent blockage of the air entry ports with substrate.

3.9.2. Fermenter set-up.

The fermenter was filled with a 3 litre volume of bulgar wheat wetted and equilibrated to a_w 0.990. A 2 litre conical flask was utilised as the air humidification vessel. This was filled with 1 litre of a glycerol/water solution at 0.970 a_w and sealed with a rubber bung containing air entry and exit ports. The fermenter and humidification bottle were autoclaved twice over subsequent days (121°C, 20 min.) at the facilities of TerraGen Discovery Ltd. in an SEC S/E autoclave (SEC Ltd., Sutton in Ashfield, Notts. UK.). The processes were controlled by a sensor positioned in the centre of a second identical “dummy” fermenter to ensure that the autoclave cycles were adequate for the entire substrate load. The sterilised fermenter and humidification vessels were coupled together using sterilised silicone rubber tubing with in-line air filters as shown in Figure 3.3.

3.9.3. Inoculation and incubation.

Preparation of the inoculum was as described in Section 3.5.4. 80g of inoculum was added directly via the lid port making an overall addition of approx. 5 %. Inoculation was carried out in a laminar flow cabinet. Air flow rates were governed and monitored by rotameters as described in Section 3.6.1. Initial entry air flow rate was set to 3 litre min^{-1} . The apparatus was run for 5 days prior to inoculation to allow fungal or bacterial contamination to develop, if present. The fermenter was then run for 14 days after inoculation at 25°C with diurnal lighting. Internal and ambient temperature, air flow rates and air a_w were monitored manually throughout, although problems occurred with measurement of air a_w .

3.9.4. Sampling and secondary metabolite analysis.

At the end of the study material was removed from the fermenter in three equally sized sections: “top”, “middle” and “bottom”. Three replicate samples were taken from each of these for analysis. The remaining material from the three sections was then pooled to form a bulk sample, and three replicate samples was taken from this also. All samples were 40cm³ volume. Metabolite analysis was as described in Sections 3.4.7. and 3.4.8.

3.10. THIRD LABORATORY SCALE FERMENTER STUDY.

Following on from the initial study utilising a modified Bioengineering laboratory scale fermenter, two further experiments were performed using the same basic

fermenter unit with provision for mechanical agitation of the substrate. This was primarily to distribute the inoculum uniformly, and possibly provide mixing during the course of the fermentation. A number of stirrer designs and arrangements were attempted in an effort to overcome the various problems involved in efficiently stirring the solid substrate mass.

3.10.1. Fermenter design.

For these experiments the Bioengineering fermenter was set-up essentially as described in Section 3.9.1. However, the aeration system was re-configured such that entry air was introduced into the headspace via a single sinter glass unit in the lid section. The fermenter unit was also turned through 90° in use and run effectively “on its side” as a “tunnel” type bioreactor (see Fig. 3.4.). Exit air was removed from the vessel via two sinter glass units mounted in the base of the fermenter (i.e. at the bottom of the substrate column). Provision was also made for dry sterile compressed air to be diverted directly into the fermenter unit without prior humidification. The fermenter was further modified with a number of stirrer configurations to attempt to introduce substrate agitation into the system. The arrangements attempted were as follows:

1. The standard Bioengineering stirrer motor and shaft unit with two open turbine (Rushton) type impellers.
2. The standard Bioengineering shaft unit modified to run on an electric drill (Bosch) with two oversize open turbine impellers.

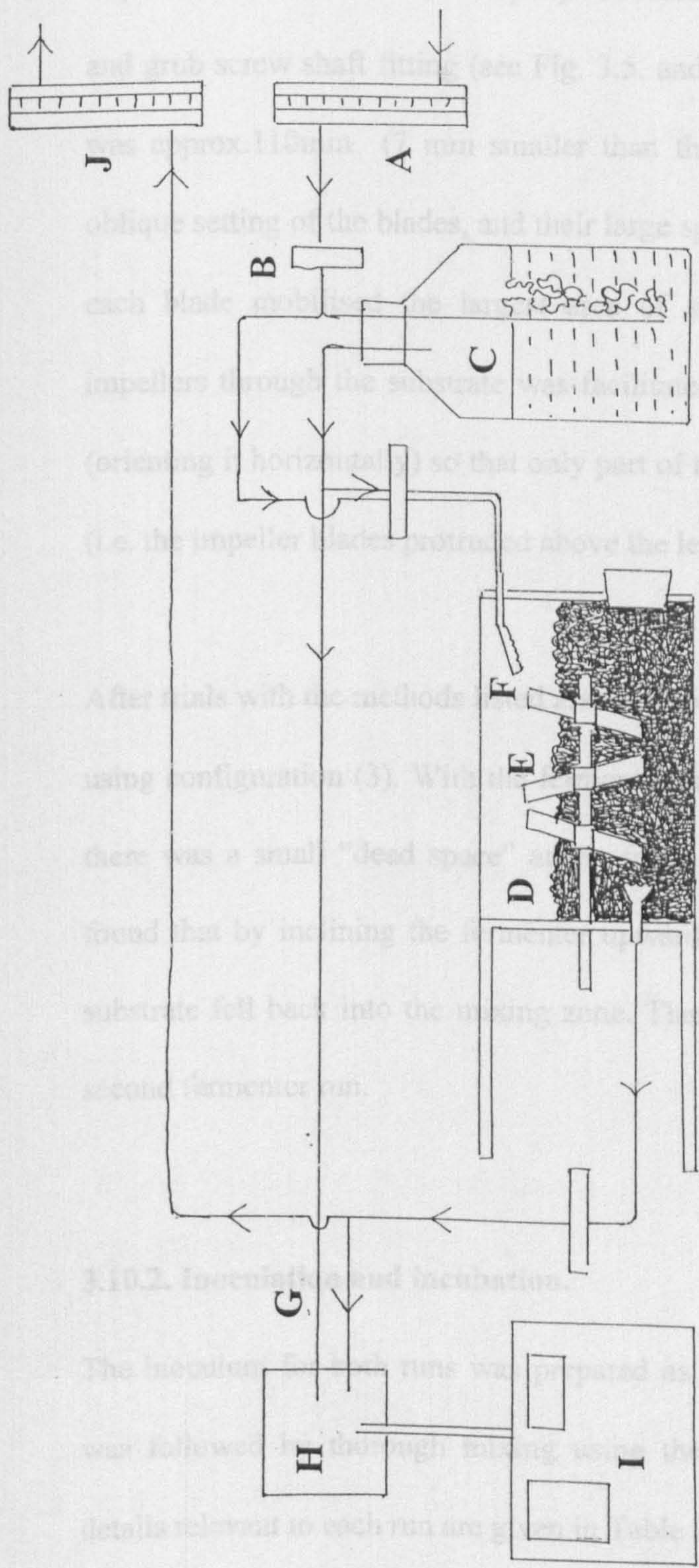


Figure 3.4. Schematic diagram of horizontally oriented fermentation apparatus incorporating mechanical agitation. A – input air rotameter,

B – air filter, C – humidifier, D – fermenter, E – stirrer, F – sinter glass air entry port, G – air a_w measurement lines, H – a_w sensor, I – a_w meter, J – output rotameter.

3. The same modified shaft as (2) fitted with three “custom” made impellers. The impellers consisted of two obliquely set stainless steel fins mounted on a central ring and grub screw shaft fitting (see Fig. 3.5. and Plate 3.3.). The overall impeller width was approx. 110mm (7 mm smaller than the overall glass column diameter). The oblique setting of the blades, and their large span was designed so that the “sweep” of each blade mobilised the largest area of substrate possible. The passage of the impellers through the substrate was facilitated by turning the fermenter through 90° (orienting it horizontally) so that only part of the impeller travel was through the solid (i.e. the impeller blades protruded above the level of the bed).

After trials with the methods listed above, two separate fermenter runs were attempted using configuration (3). With the fermenter unit arranged horizontally during stirring there was a small “dead space” at the front that was not mixed adequately. It was found that by inclining the fermenter upwards by about 45° from the horizontal the substrate fell back into the mixing zone. Therefore this orientation was used for the second fermenter run.

3.10.2. Inoculation and incubation.

The inoculum for both runs was prepared as described in Section 3.5.4. Inoculation was followed by thorough mixing using the stirrer apparatus. Specific incubation details relevant to each run are given in Table 3.6.

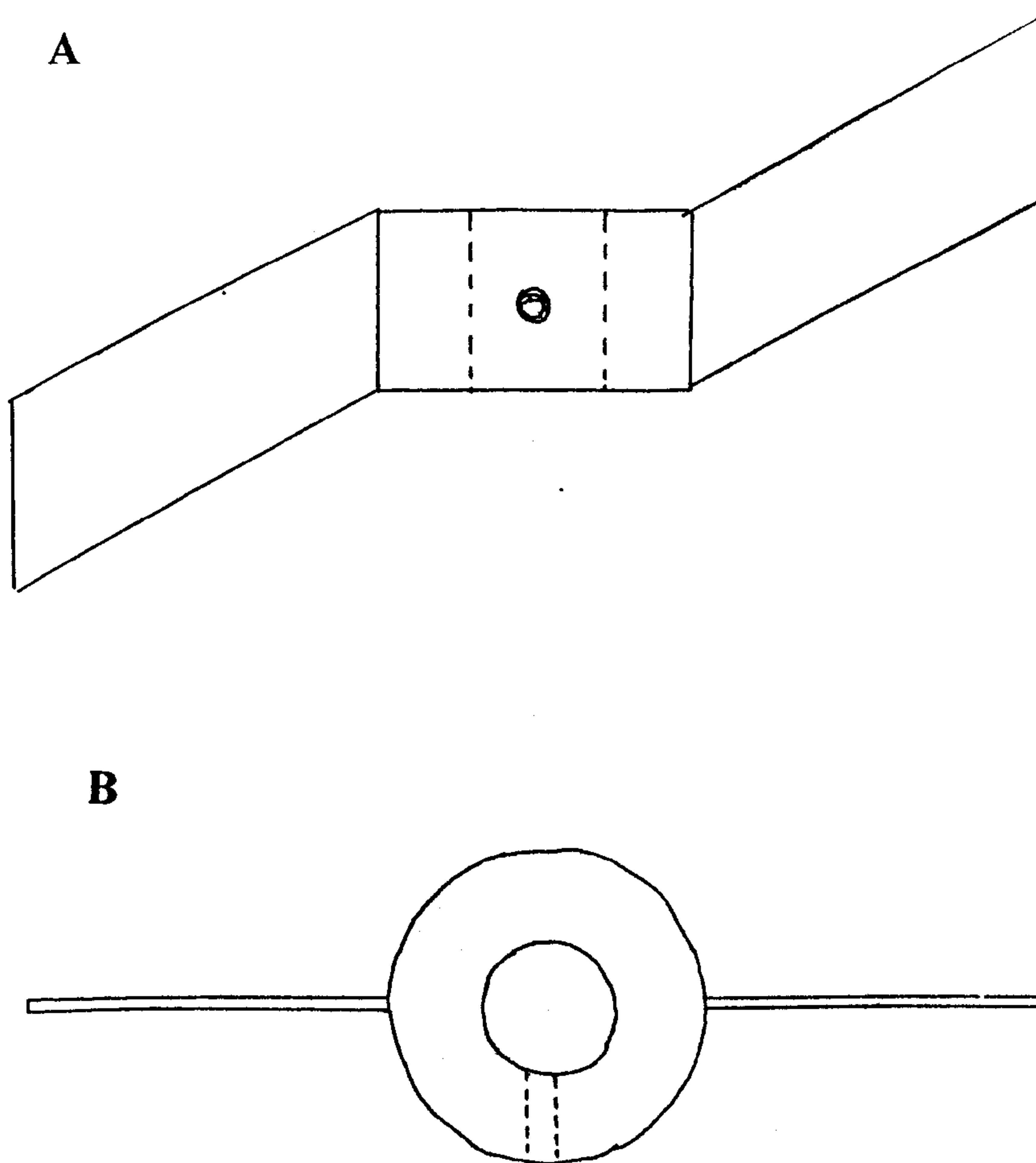


Figure 3.5. Purpose made impeller unit (scale 1:1). A – side elevation , B – plan view.

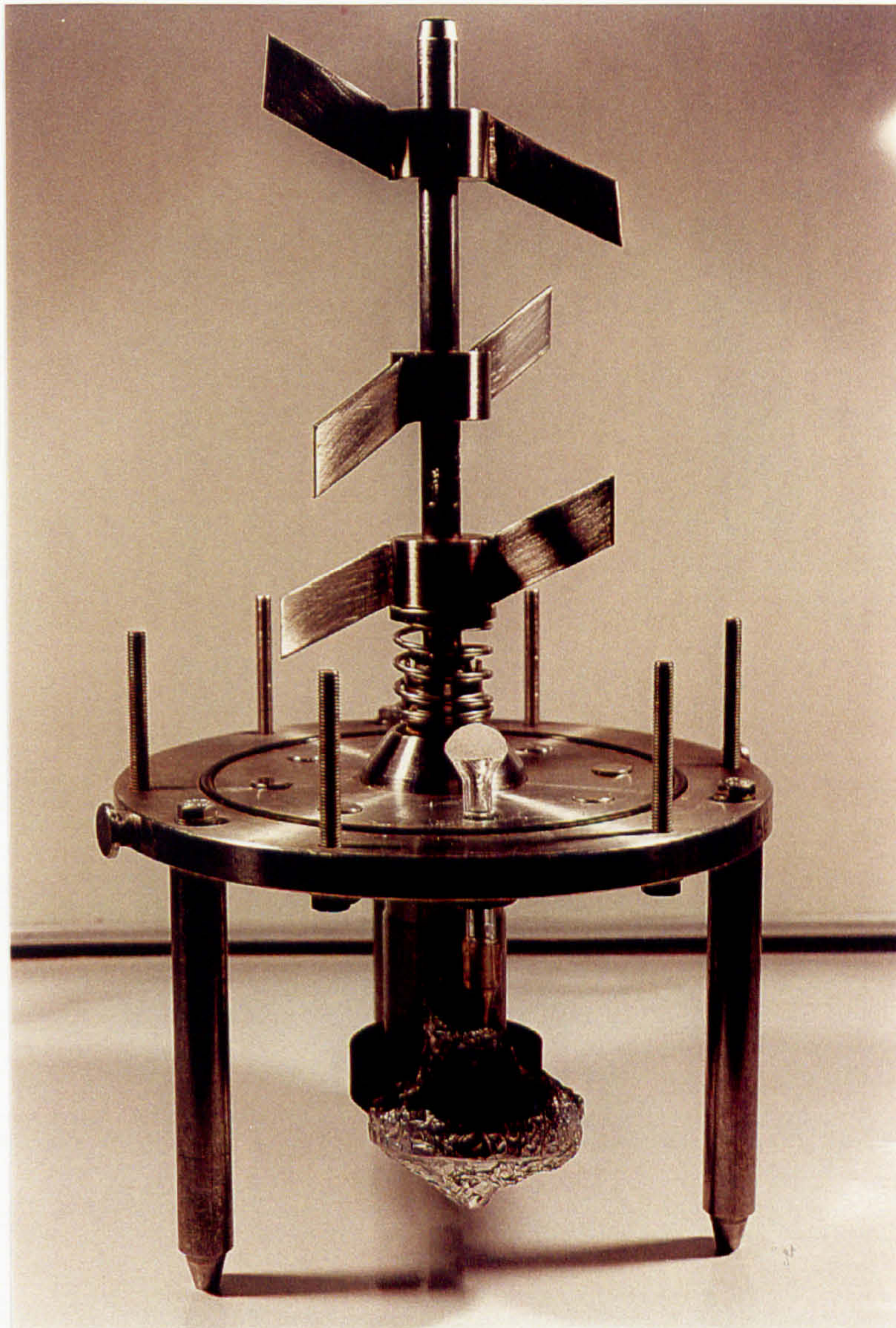


Plate 3.3. Fermenter base unit showing stirrer shaft and impellers.

3.10.3. Sampling and secondary metabolite analysis.

Sampling details are given in Table 3.6. Sample volumes were 40 cm³ in all cases.

Secondary metabolite analysis was as described in Sections 3.4.7. and 3.4.8.

3.11. TREATMENT OF RESULTS.

3.11.1. Moisture sorption isotherms.

Required moisture levels in grain were derived from the equation :

$$x = \text{weight of grain. } (y - a) / 100 - y$$

where: x = amount of water required to add

a = initial water content (%) of grain

y = required water content (%)

Moisture contents of grain (%, wet weight basis) were calculated from the equation :

$$\% \text{ moisture} = \text{weight loss} / \text{initial wet weight} \times 100/1$$

where: weight loss = (wet weight sample + beaker) - (dry weight sample + beaker)

initial wet weight = (initial weight + beaker) - (weight beaker).

Water activity (a_w) was calculated from Humidat readings from the equation :

$$a_w = \text{reading } (\% \text{ ERH}) / 100$$

3.11.2. *Phoma* growth studies.

Colony growth rates were calculated from:

1. Colony radius = colony diameter / 2

Table 3.6. Summary of horizontally oriented fermenter runs.

Run No.	1	2
Inoculum level	100g	100g
Substrate/humidifier a_w	0.970/0.970	0.970/0.900
Substrate volume (wet)	3 litre	3 litre
Agitation regime	T.0 and intermittent, once daily 30 sec.	T.0 only, to distribute inoculum.
Aeration regime	2 l min ⁻¹ humid air, intermittent dry air.	2 l min ⁻¹ humid air
Fermenter orientation	Horizontal	Horizontal, inclined upwards
Temperature	25°C	25°C
Run duration (days)	7	14
Sampling (time (day) and type)	7: front, middle, back.	7: average 14: Top, bottom.
Additional comments	-	0.01% chloramphenicol (wt/wt) added to substrate.

2. The colony radial extension (mm) against time (days) was plotted for each replicate.

3. Colony radial growth rates (mm day⁻¹) were then calculated (Microsoft Excel version 5.0) from the linear portion of curves derived in 2. above.

3.11.3. *Phoma* metabolite studies - HPLC results.

Analysis of HPLC results was via dedicated Gilson 715 HPLC system controller software giving initial results in µg cm⁻³ extract against the calibration curve constructed for each run. For samples reported as “high” or “low” by the software results were obtained from the calibration curve using the equation :

$$y = a + bx$$

re-arranging :

$$x = y - a / b$$

where : a = intercept

b = slope of line

Results obtained in µg ml⁻¹ extraction solvent were converted to µg g⁻¹ wet weight agar from the equation :

$$\text{Yield} = (\text{yield } \mu\text{g S1/plug weight}) \times (100 / \text{extraction efficiency } (\%))$$

Extraction efficiency (%) was derived from the equation :

$$(\text{Yield } \mu\text{g ml}^{-1} / \text{Theoretical yield } \mu\text{g cm}^{-3}) \times (100 / 1)$$

3.11.4. HPLC results for other fungal species.

Analysis of HPLC results was via Waters “millennium” software, which supplied a printed report for each sample (see appendix 2). The report included spectrum index plots for each peak, and a peak results table. Spectrum index plots together with retention times were used to identify metabolites, and peak area, measured in absorption units (UV * sec), was used to quantify metabolites. All samples were run in triplicate to allow statistical analysis.

3.11.5. Error bars on graphs.

Error bars are standard errors about the mean given by the expression:

Error = standard deviation / square root of No. of replicates (n).

3.11.6. Statistical analysis

Analysis of variance (ANOVA): ANOVA's were carried out using Microsoft Excel tool package. Single factor and two factor tests were used as appropriate. Two factor with replication was used to investigate the effect of two variables in each experiment, and the effect of interaction between these. ANOVA's were carried out at the 5% significance level.

Least significant difference (LSD): LSD analysis was carried out on data following ANOVA to identify significant differences between individual planned treatments.

LSD's were derived from the expression:

$LSD = \text{square root} (\text{within group Mean Square} \times 2 / \text{replicate per treatment}) \times t_x$

where $t_x = t$ distribution value at x degrees of freedom

and $x =$ degrees of freedom within treatments.

The LSD value was then applied to individual pairs of means (i.e. $x - y$) to establish whether a significant difference existed (i.e. where $x - y > LSD$).

LSD tests were considered valid since these were applied to planned comparisons only, and not comparisons that were suggested by the data after the experiment. In particular, comparisons of all possible treatment means were never considered in any experiment.

Tables of statistical analyses were shown where appropriate. These showed the values of F critical (F_{crit}) and F obtained in the tests, and allowed assessment of the extent of significance of results by comparison of these two figures (i.e. if F was less than, or equal to F_{crit} , no significant difference existed).

CHAPTER 4

RESULTS

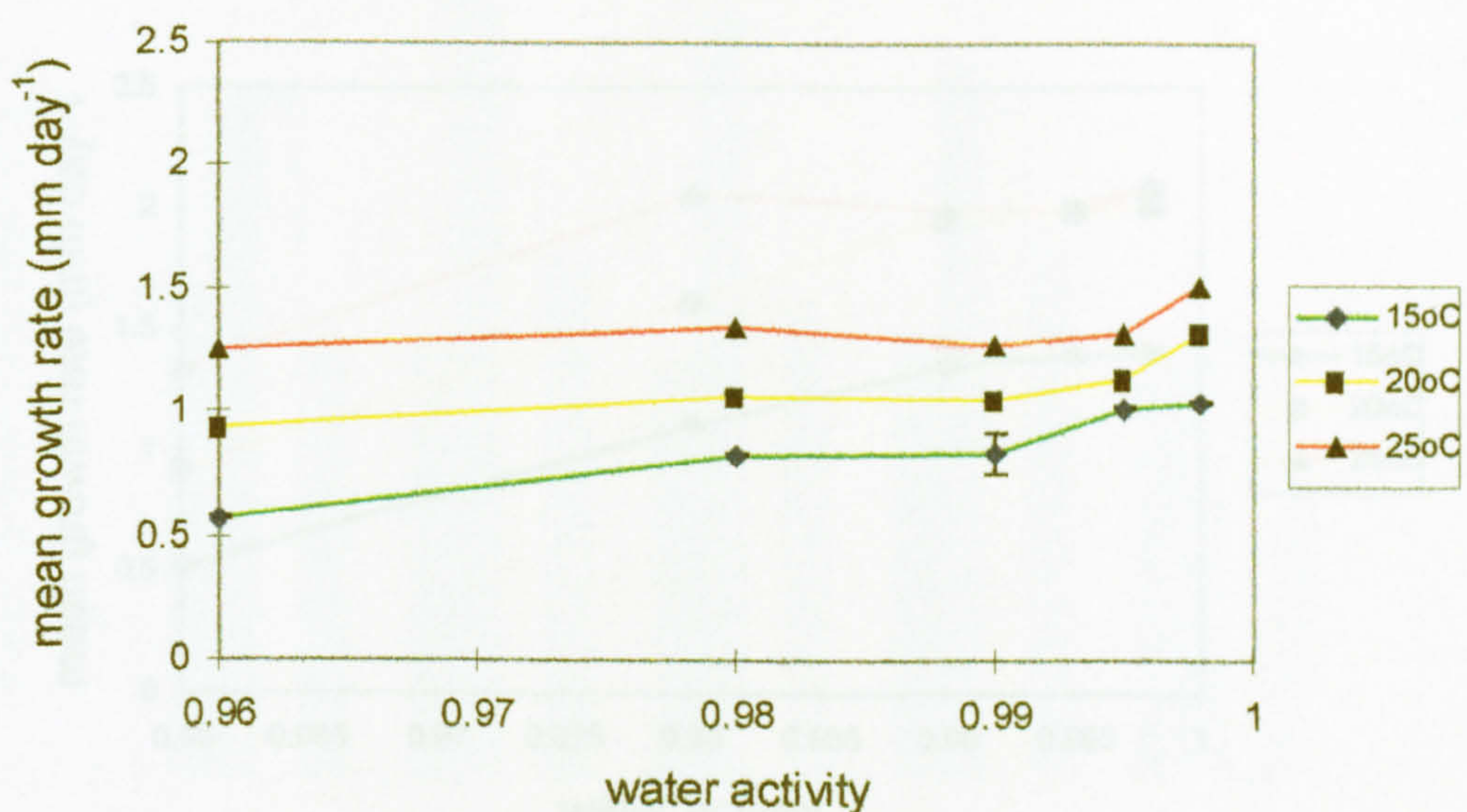
4.1. GROWTH AND SECONDARY METABOLITE STUDIES WITH A *PHOMA* SPECIES USING GRAIN AND SEED EXTRACTS.

4.1.1. Effects of substrate type, a_w and temperature on growth.

The effect of interaction between a_w and temperature on linear growth rate of the *Phoma* species on MEA, WEA, OEA and OSREA is shown in Figure 4.1. This shows a marked effect of temperature on linear growth rate when compared to the effect of a_w over the range investigated. The data for 15, 20 and 25°C are separated into three distinct groups in each case with statistically significant differences ($P = 0.05$) between linear growth rates at each temperature for a given a_w level. The only exception to this is shown in OEA where the 20 and 25°C growth rates are similar above 0.99 a_w . The effect of a_w on linear growth rate was also statistically significant, but had less influence than temperature as demonstrated by the ANOVA data (Table 4.1.). The growth rate was significantly reduced at 0.96 a_w for all media and temperatures, except for MEA at 25°C ($P = 0.05$).

Maximum growth rates for all media types were recorded at 25°C. The influence of medium type and a_w on mean growth rate at 25°C is shown in Figure 4.2. For grain-derived and seed-derived media, growth rates were comparable for WEA and OEA, and significantly lower for MEA and OSREA (OSREA appeared to be a very poor growth substrate for this organism). Linear growth rates on MEA were significantly lower than for starchy grain derived media, but different growth forms were noted growing on these two media types. On MEA a very dense growth form was noted,

a. Malt extract agar.



b. Wheat extract agar.

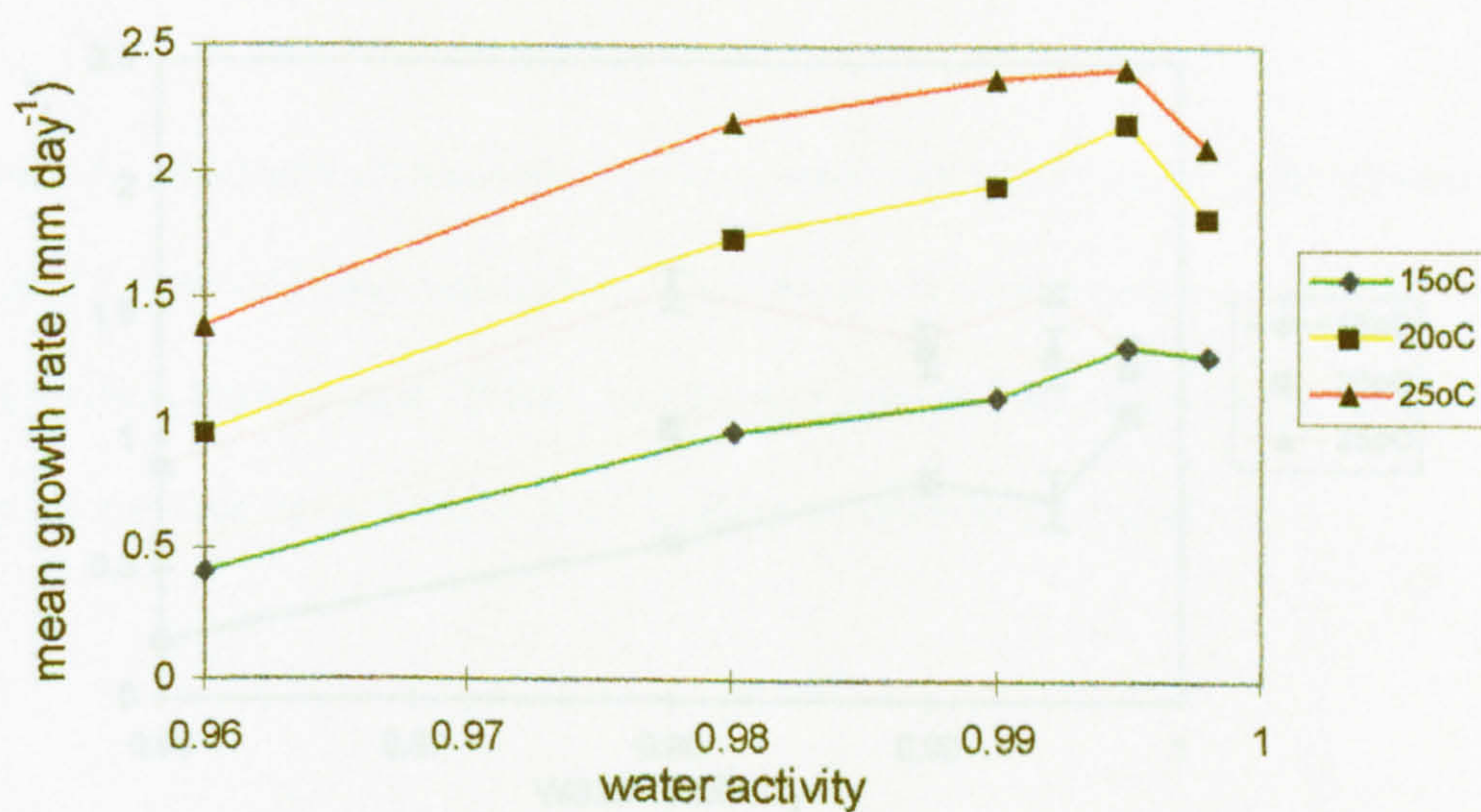
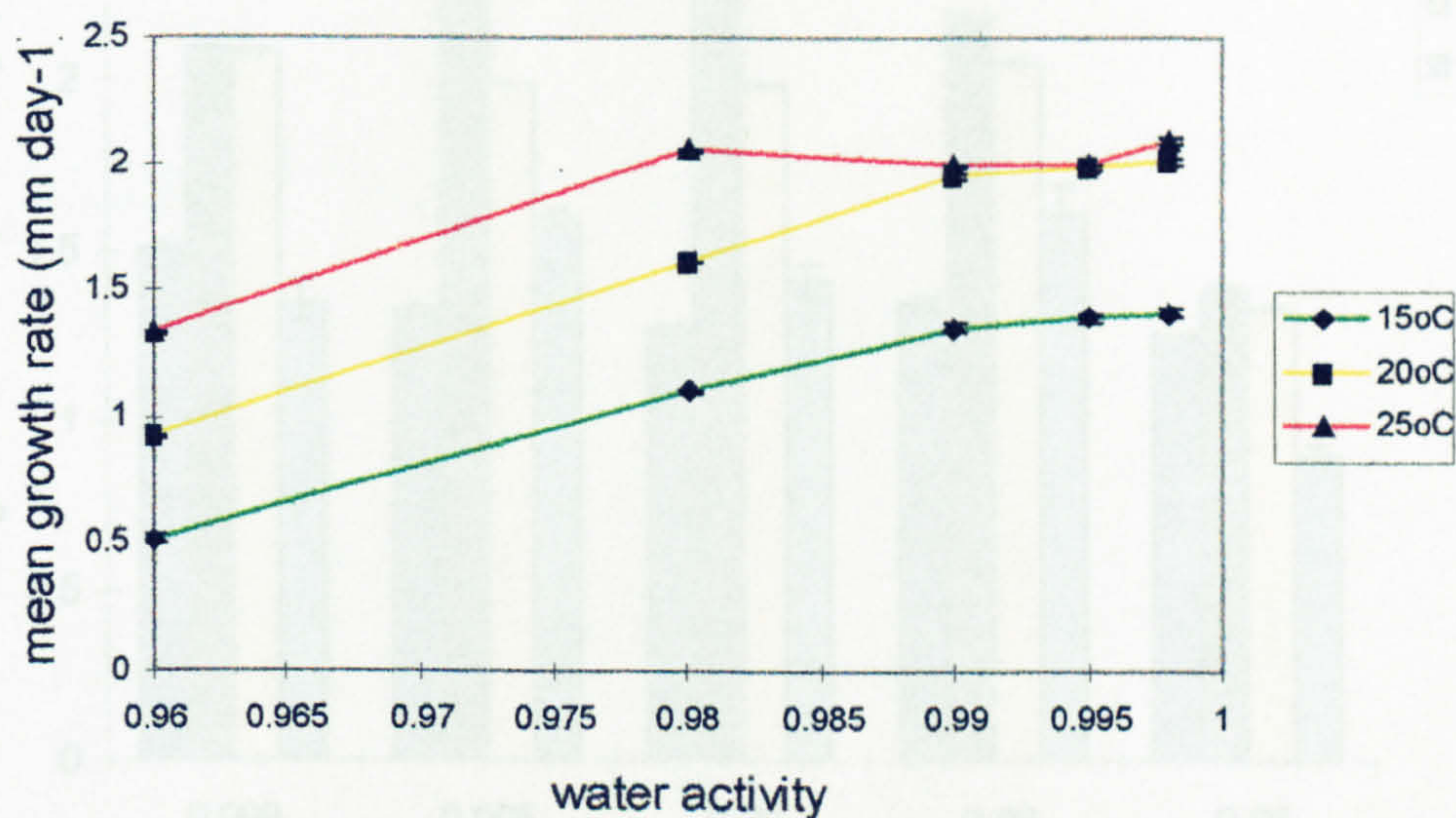


Figure 4.1. Influence of temperature and water activity (a_w) on mean growth rate of *Phoma* sp. (mean of three replicates). Standard error bars are shown where larger than symbol size.

c. Oat extract agar.



d. Oil seed rape extract agar.

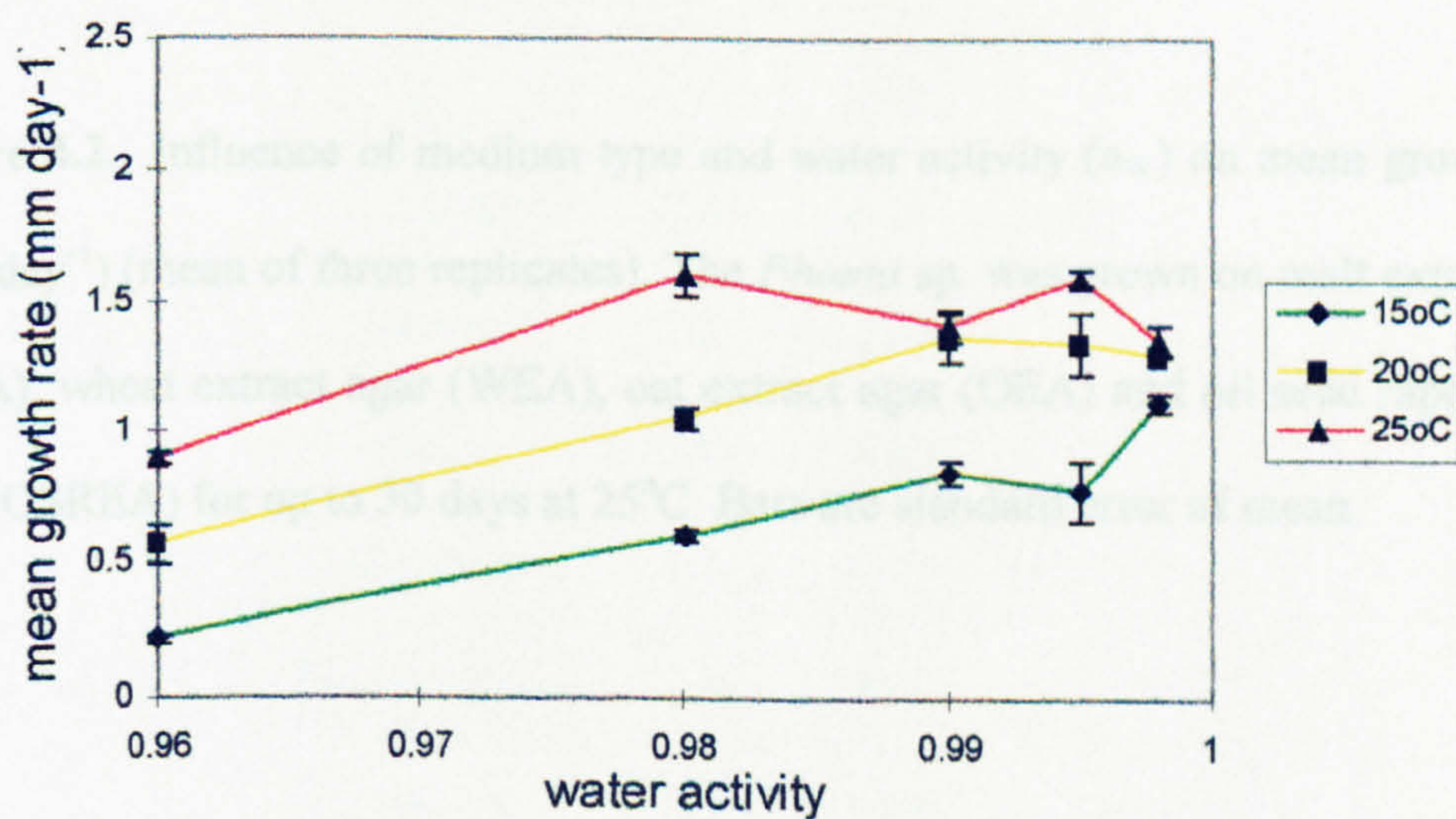


Figure 4.1. (cont.) Influence of temperature and water activity (a_w) on mean growth rate of *Phoma* sp. (mean of three replicates). Standard error bars are shown where larger than symbol size.

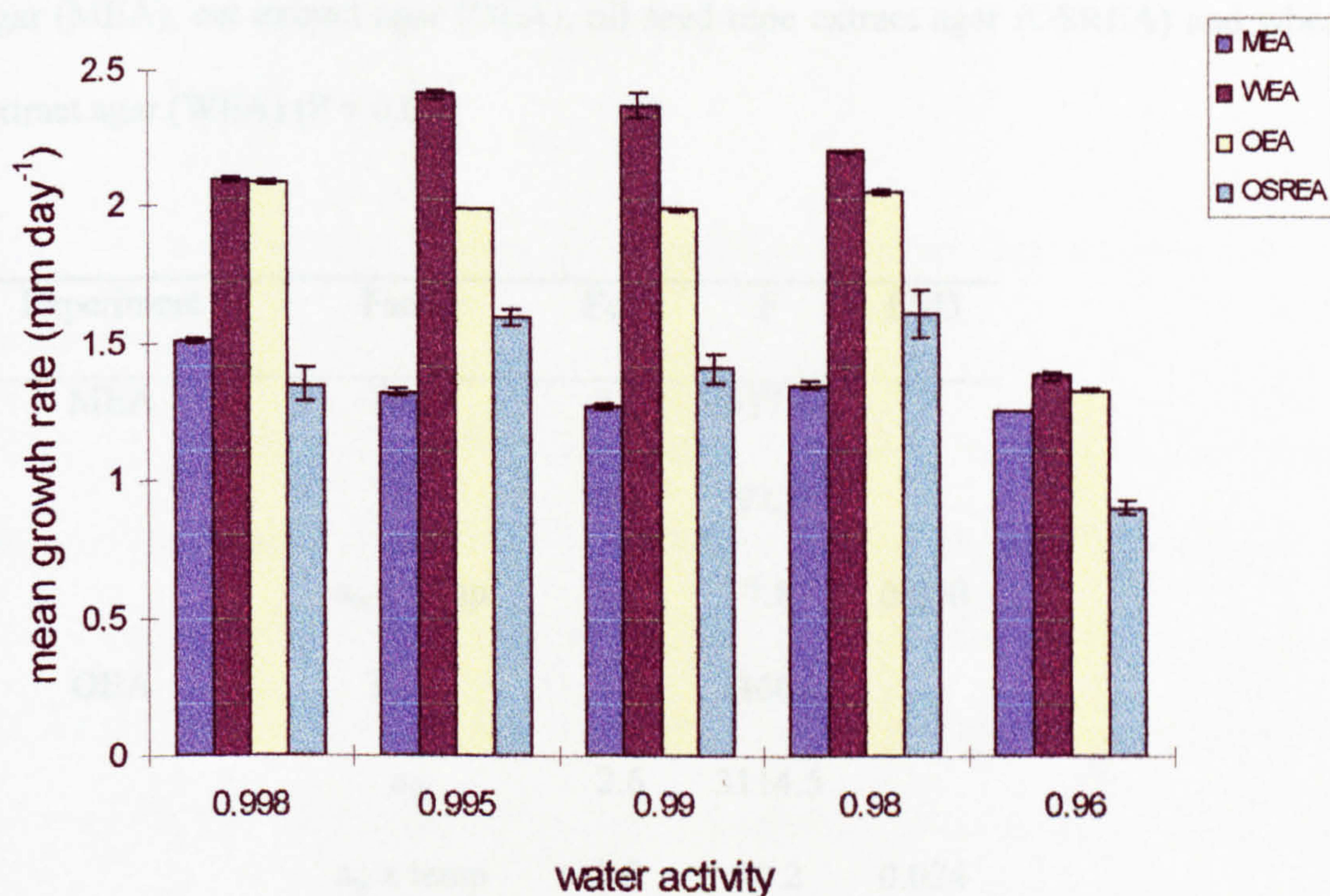


Figure 4.2. Influence of medium type and water activity (a_w) on mean growth rate (mm day⁻¹) (mean of three replicates). The *Phoma* sp. was grown on malt extract agar (MEA), wheat extract agar (WEA), oat extract agar (OEA) and oil seed rape extract agar (OSREA) for up to 30 days at 25°C. Bars are standard error of mean.

Table 4.1. Statistical analysis of growth data for *Phoma* sp. growing on malt extract agar (MEA), oat extract agar (OEA), oil seed rape extract agar (OSREA) and wheat extract agar (WEA) (P = 0.05).

Experiment	Factor	Fcrit	F	LSD
MEA	Temp	3.2	457.4	
	a _w	2.6	92.6	
	a _w x temp	2.2	7.8	0.050
OEA	Temp	3.2	5300.9	
	a _w	2.6	3114.5	
	a _w x temp	2.2	90.2	0.024
OSREA	Temp	3.1	126.0	
	a _w	2.5	58.9	
	a _w x temp	2.0	5.8	0.132
WEA	Temp	3.2	6137.8	
	a _w	2.6	2093.2	
	a _w x temp	2.2	46.9	0.031
All media, 25°C	Medium type	2.8	495.8	
	a _w	2.5	190.9	
	Med type x a _w	1.9	23.6	0.075

while on the grain derived media a very diffuse growth form was evident (see Plate 4.1.).

4.1.2. Effects of substrate type, a_w and temperature on temporal squalastatin production.

Figure 4.3. shows the effects of the interaction between a_w and time on squalastatin (S1) production for all media types at 25°C. The highest yields of S1 were recorded at 30 days incubation irrespective of a_w level. This result was reflected in all experiments carried out for each medium type and temperature. It is clear from comparison of these results that S1 production on MEA, apart from being markedly lower, was delayed at days 9 and 20 in comparison to WEA. This may represent a catabolite repression effect operating for the nutritionally rich MEA, which is less likely to appear in the nutritionally depleted grain extract media. The broad differences in nutritional status between the media types were also apparent in the colony growth forms obtained.

S1 production was maximum in the a_w range 0.99 - 0.98 for WEA and OEA, 0.98 - 0.96 for MEA and 0.995 for OSREA. A similar trend was noted in all experiments carried out with overall maximum production of S1 at sub-optimal a_w levels for growth (0.99 - 0.98 a_w). Statistical tests carried out on the day 30 WEA data revealed a significant effect of a_w on S1 production ($P = 0.05$) (Table 4.2). LSD analysis confirmed significantly enhanced yields of S1 at 0.99 and 0.98 a_w , with yields at 0.98 a_w being significantly higher than any other treatments. The lowest yields were

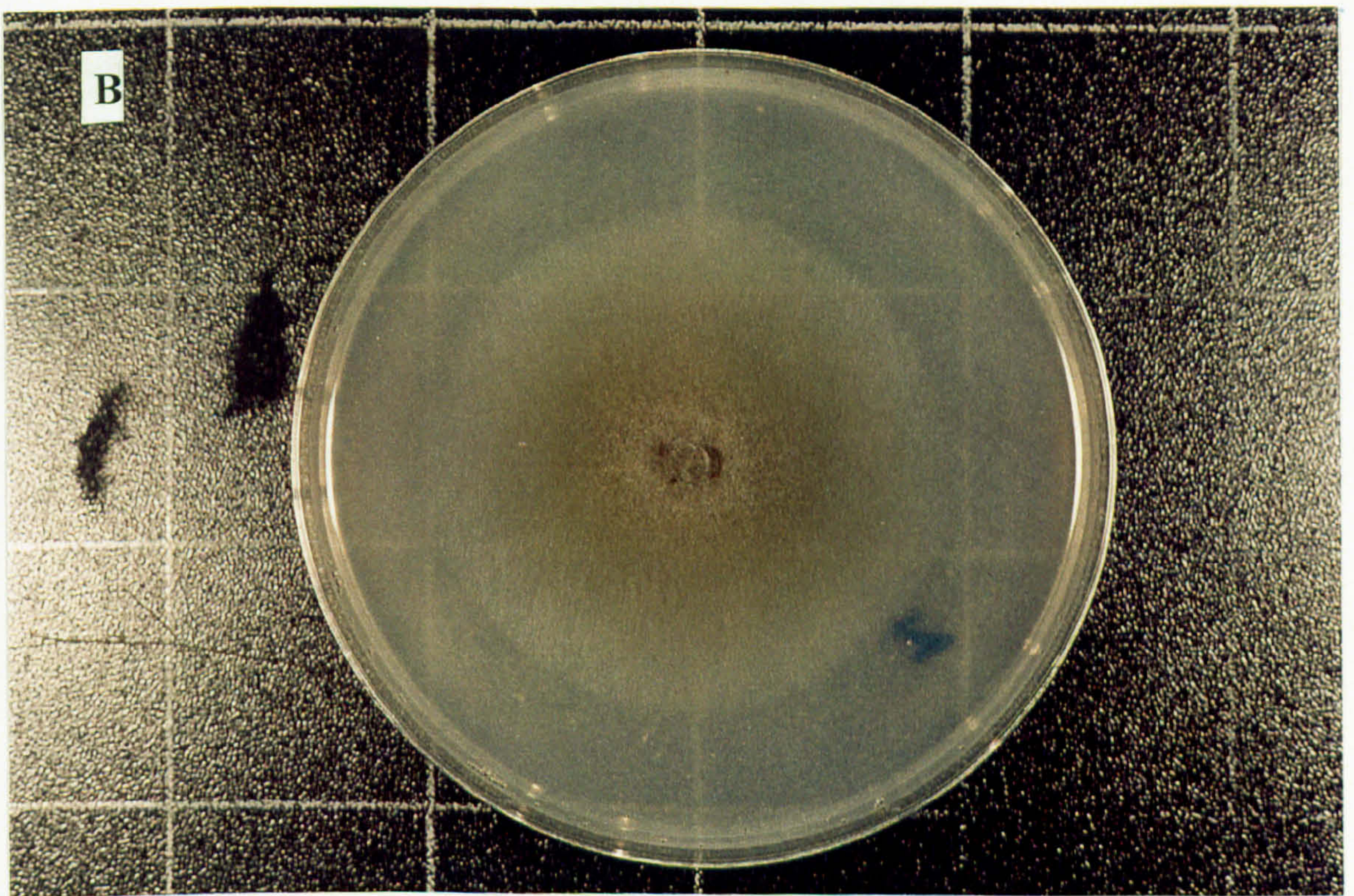
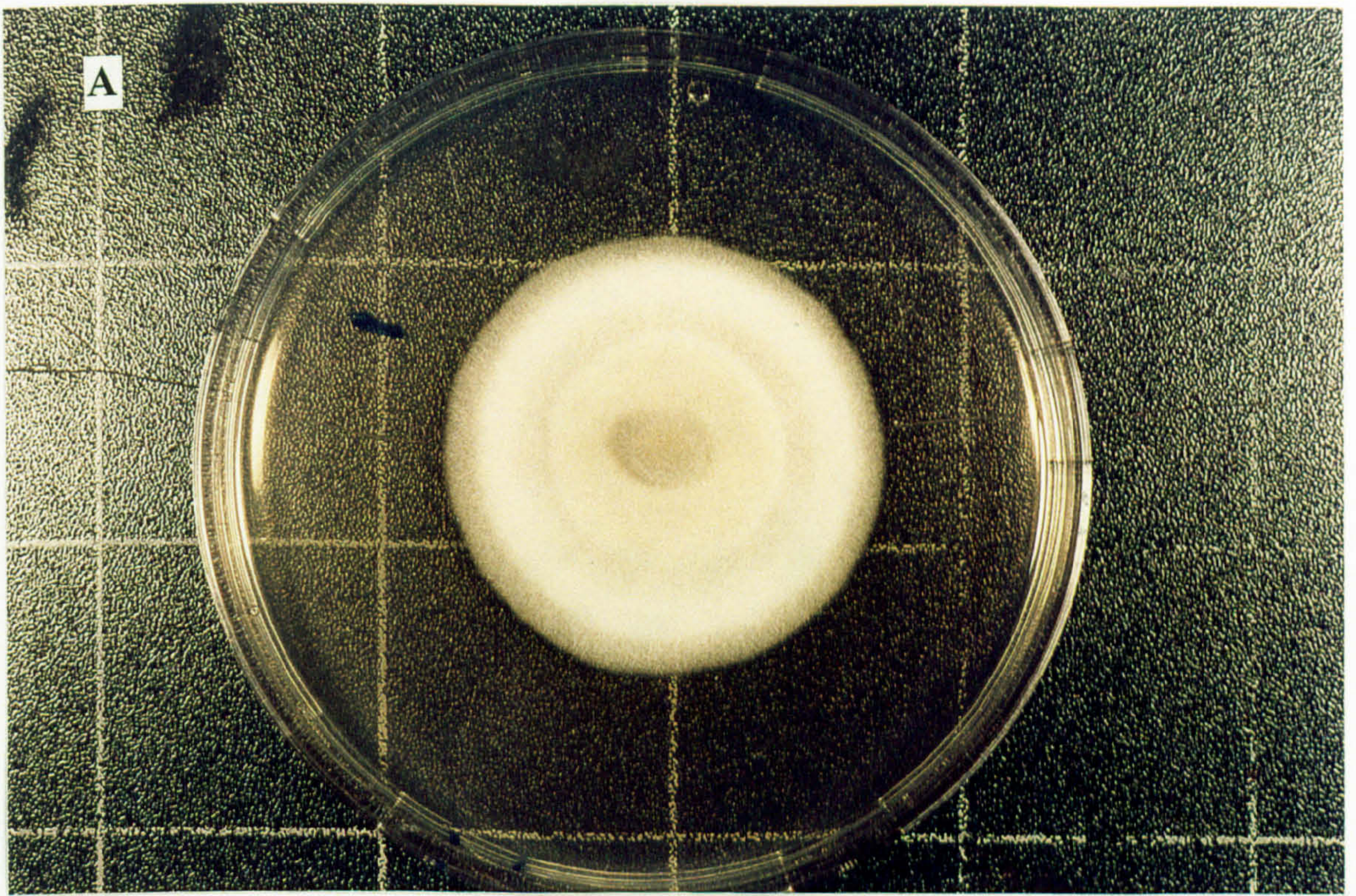
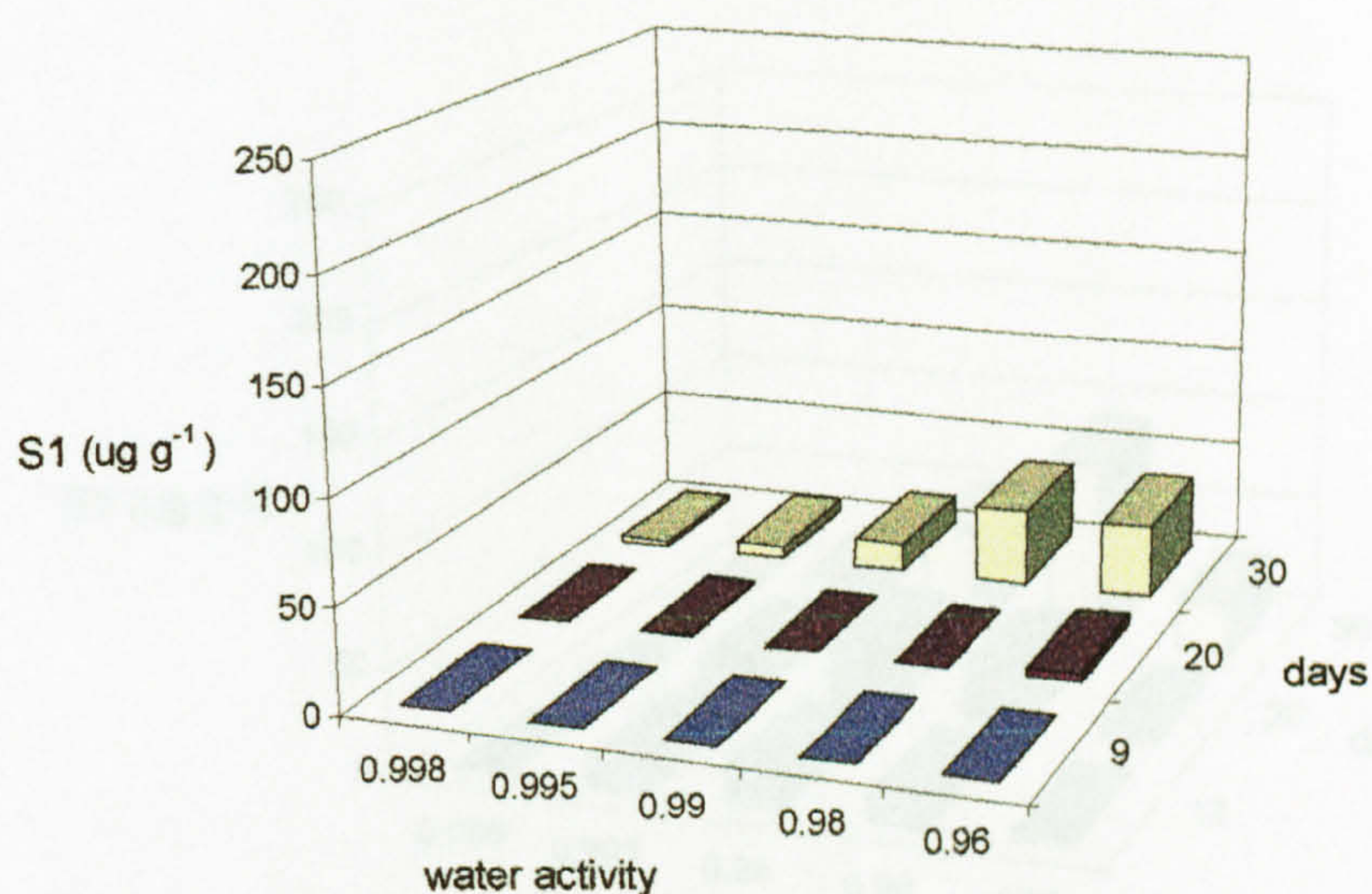


Plate 4.1. Morphology of *Phoma* species, 2 week cultures at 25°C. A – malt extract agar (MEA). B – Wheat extract agar (WEA).

a. Malt extract agar.



b. Wheat extract agar.

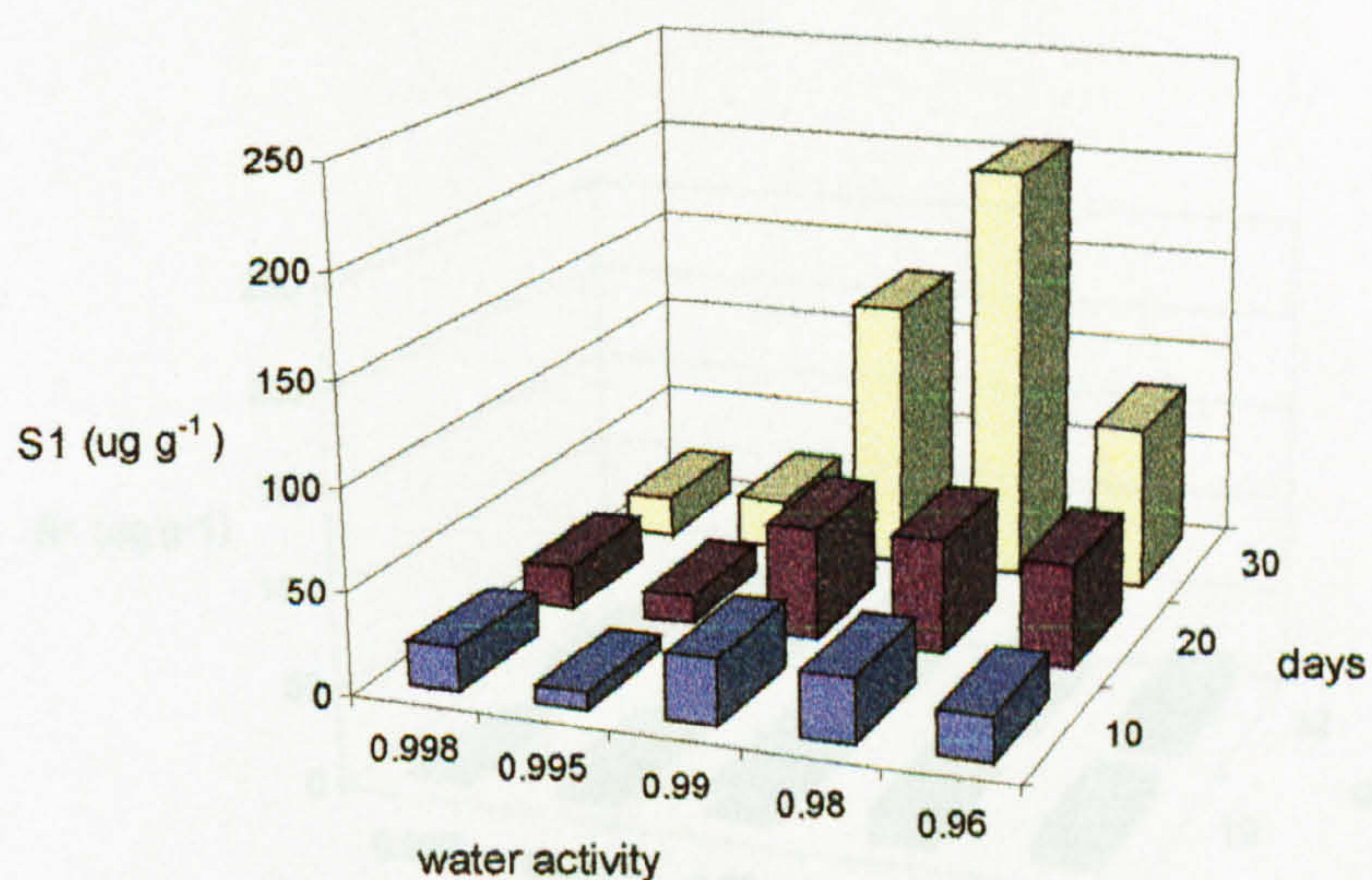
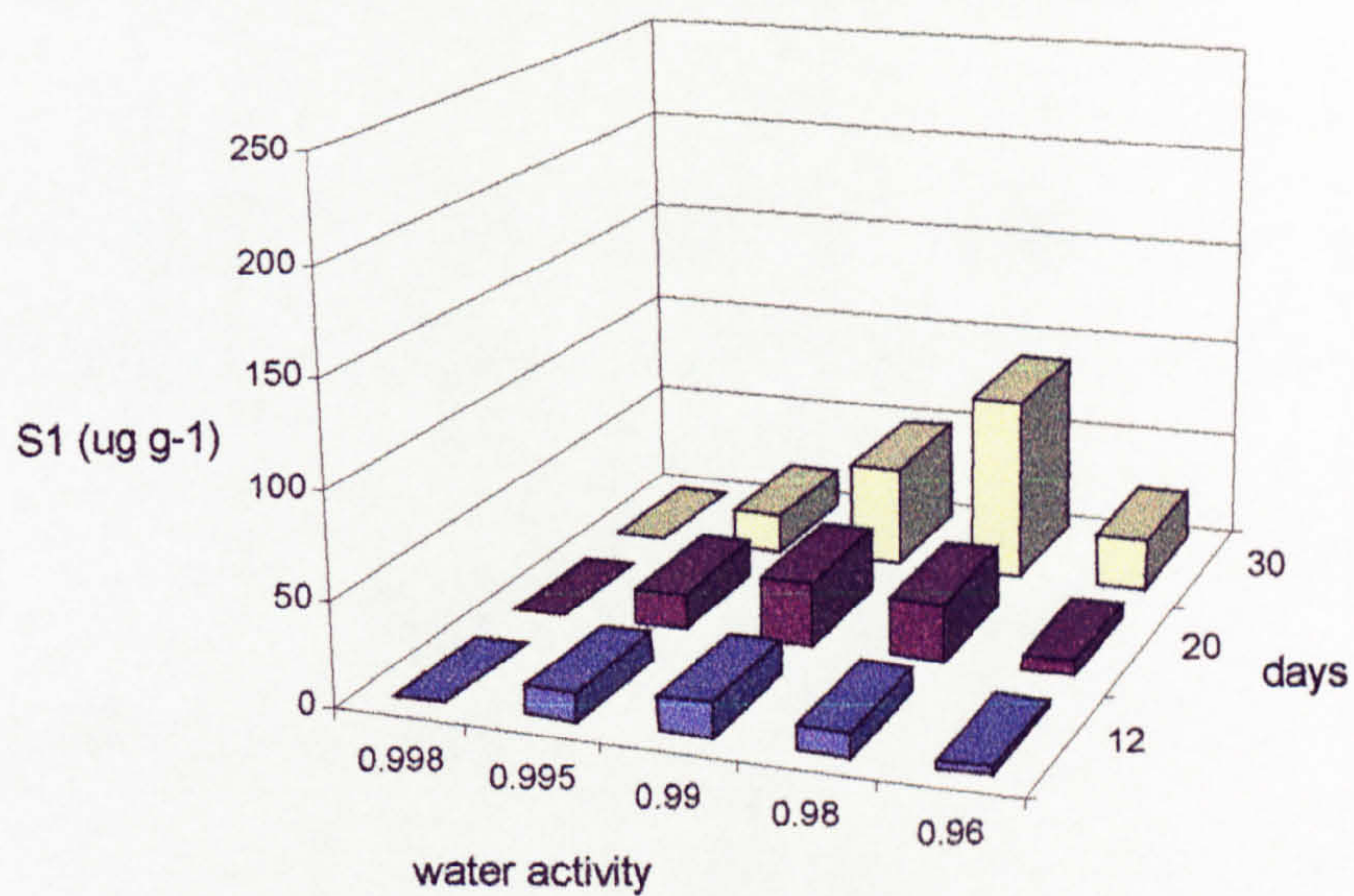


Figure 4.3. Influence of time and water activity (a_w) on mean yield of squalenstatin 1 (S1) (expressed in $\mu\text{g g}^{-1}$ wet wt. agar) (mean of three replicates) extracted from colonies of *Phoma* sp. grown for 30 days at 25°C.

c. Oat extract agar.



d. Oil seed rape extract agar.

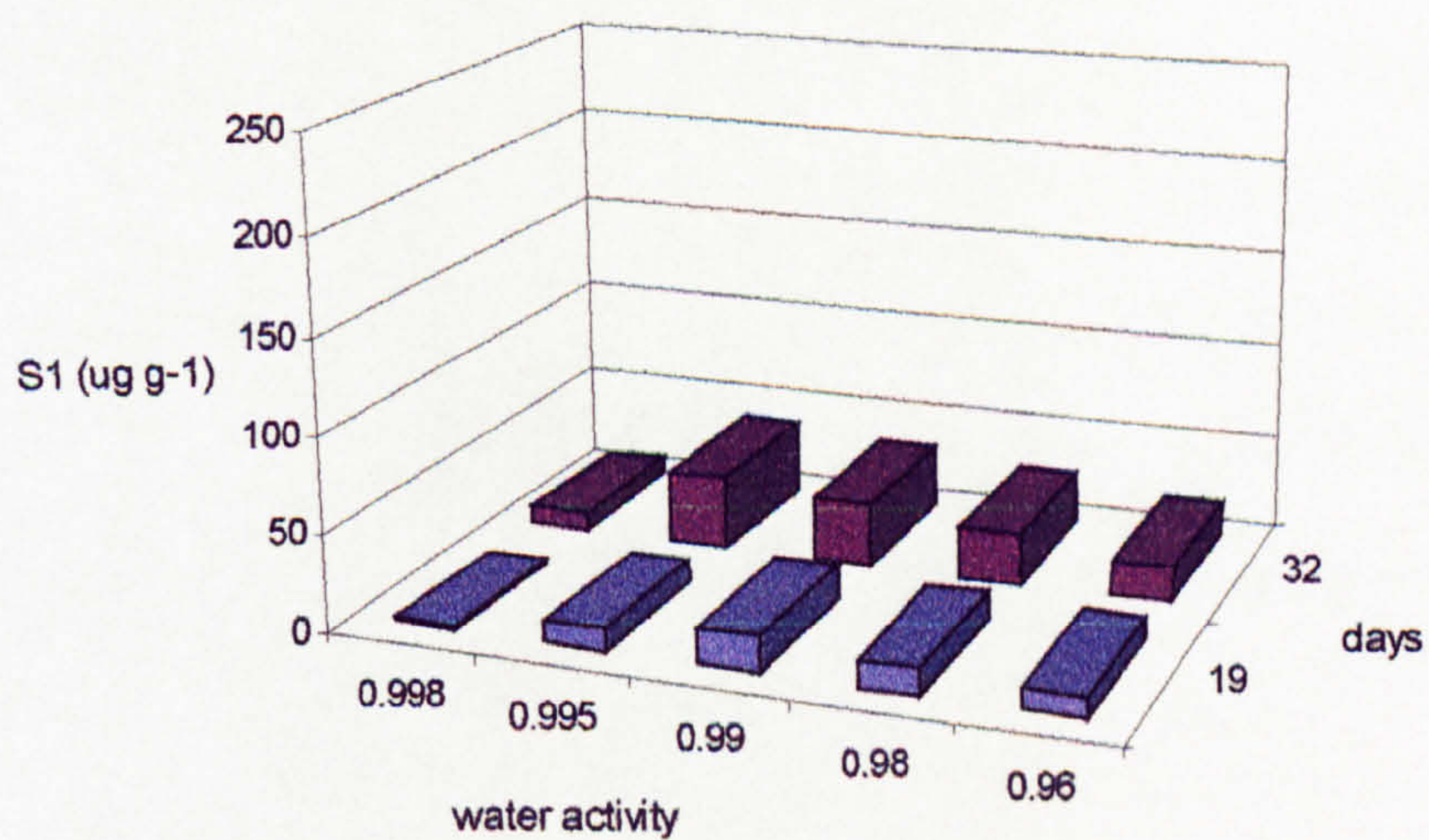


Figure 4.3 (cont.) Influence of time and water activity (a_w) on mean yield of squalstatin 1 (S1) (expressed in $\mu\text{g g}^{-1}$ wet wt. agar) (mean of three replicates) extracted from colonies of *Phoma* sp. grown for 30 days at 25°C.

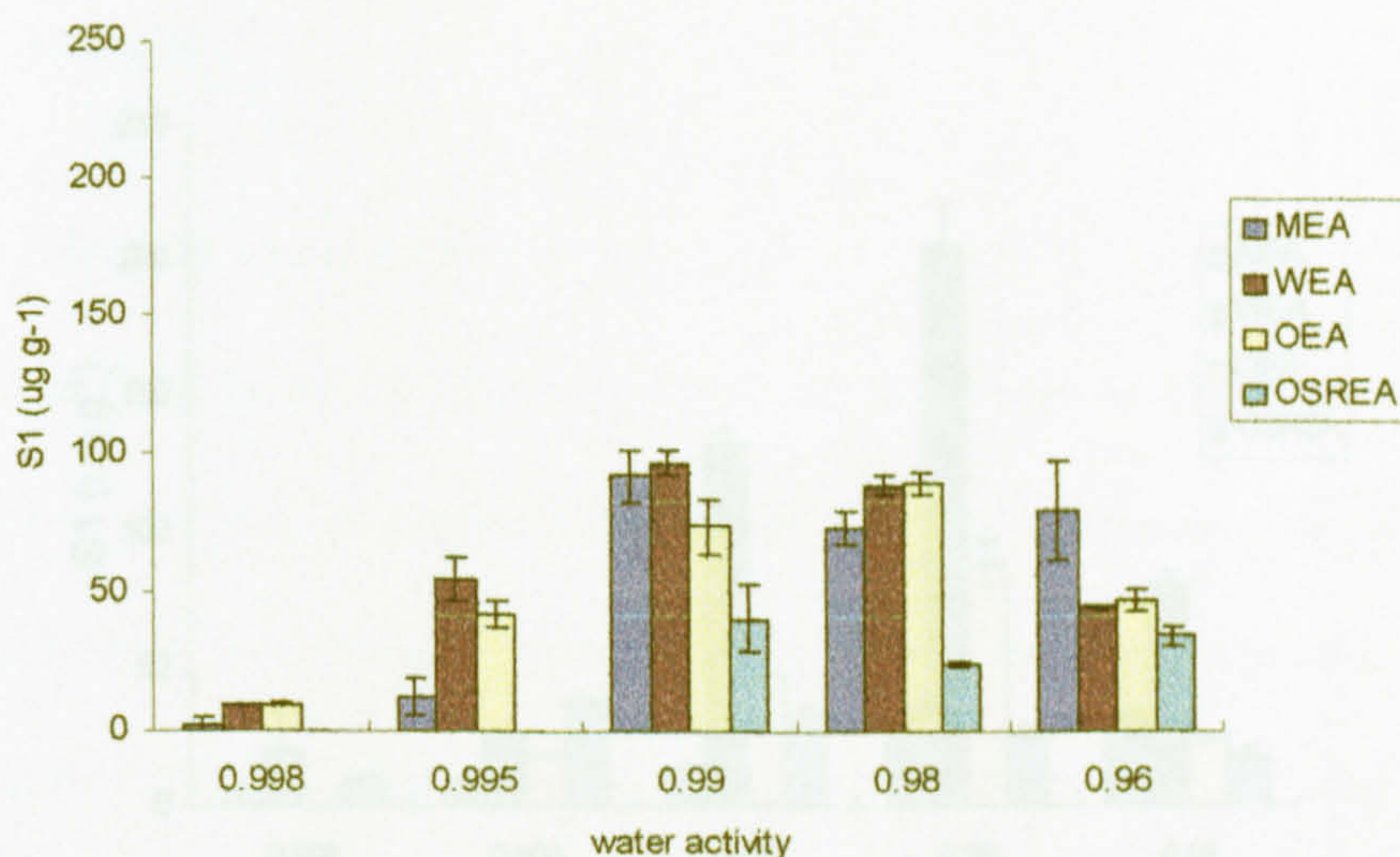
Table 4.2. Statistical analysis of metabolite production data on wheat extract agar (WEA) (day 30) and all media (day 30, 25°C) (P = 0.05).

Experiment	Factor	Fcrit	F	LSD
WEA, day 30	Temp	3.3	39.3	
	a _w	2.7	143.1	
	a _w x temp	2.3	18.3	16.1
All media, day 30	Medium	2.8	80.4	
	a _w	2.6	60.7	
	Med x a _w	2.0	18.1	16.7

observed at 0.998 a_w (when water was freely available) at all temperatures. Although ANOVA revealed a significant effect of temperature on S1 production overall, no clear-cut pattern could be discerned. For example, there was no significant difference between S1 levels at 0.998 a_w for the three incubation temperatures. At 0.995 a_w , significantly less metabolite was produced at 25°C when compared to 15°C and 20°C. At 0.98 a_w significantly more metabolite was produced at 25°C when compared to 15°C and 20°C ($P = 0.05$).

The yields of S1 at the three incubation temperatures are compared directly for all media types in Figure 4.4. The enhanced production of S1 at 0.99 and 0.98 a_w on WEA for all temperatures is particularly clear, together with the comparatively low levels produced on MEA at 25°C. OSREA was demonstrated to be the poorest substrate for the production of S1 overall. Statistical analysis of the 25°C data revealed a significant effect of media type, a_w and their interaction on S1 production ($P = 0.05$) (see Table 4.2.). In particular LSD analysis revealed that yields on WEA at 0.99 and 0.98 a_w were significantly higher than any other medium, and yields for all media at 0.99 and 0.98 a_w levels were significantly higher than yields obtained for the same media at 0.998 a_w ($P = 0.05$).

a. 15°C.



b. 20°C.

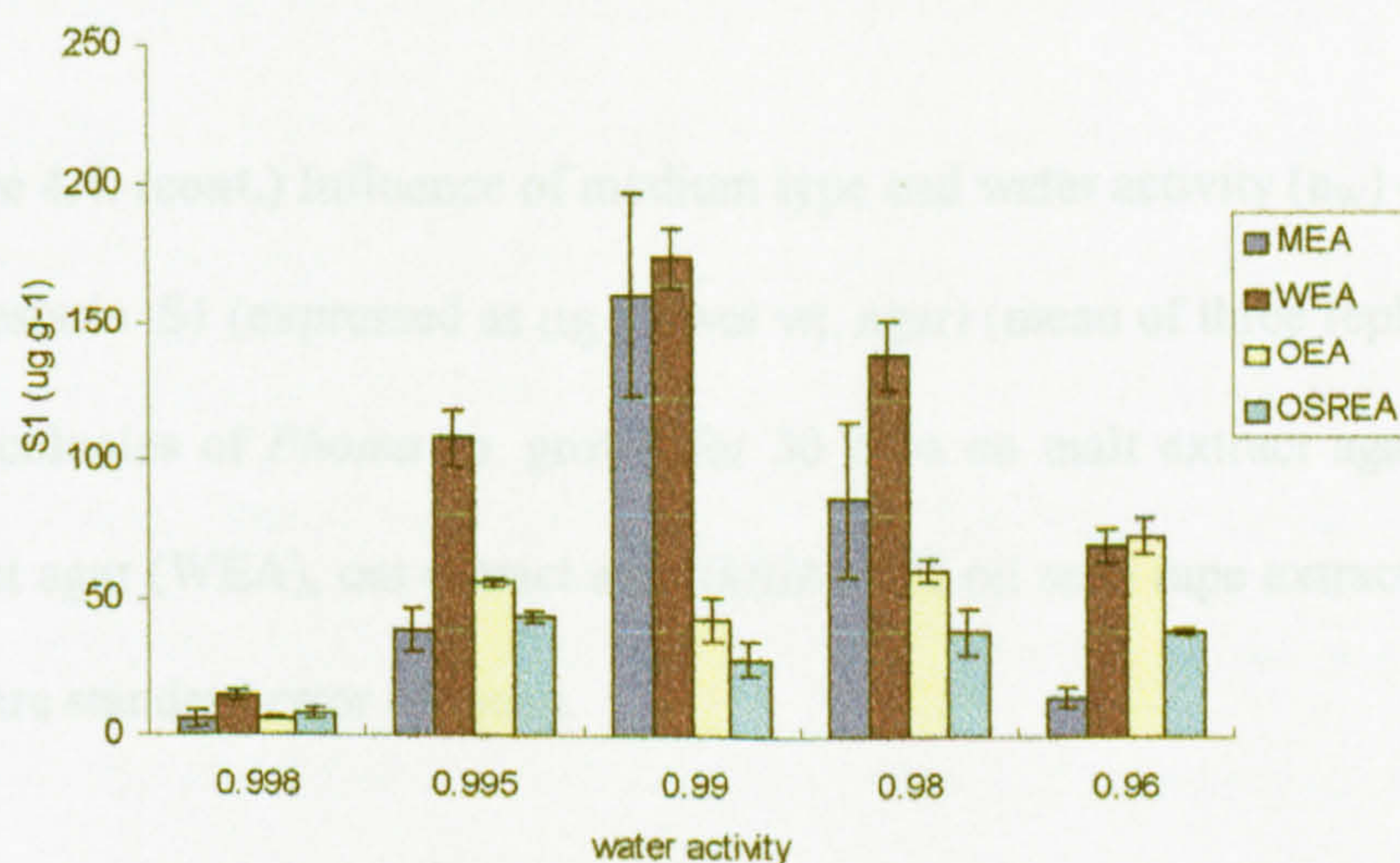


Figure 4.4. Influence of medium type and water activity (a_w) on mean yield of squalestatin S1 (expressed as $\mu\text{g g}^{-1}$ wet wt. agar) (mean of three replicates) extracted from colonies of *Phoma* sp. grown for 30 days on malt extract agar (MEA), wheat extract agar (WEA), oat extract agar (OEA) and oil seed rape extract agar (OSREA).

Bars are standard error of mean.

c. 25°C.

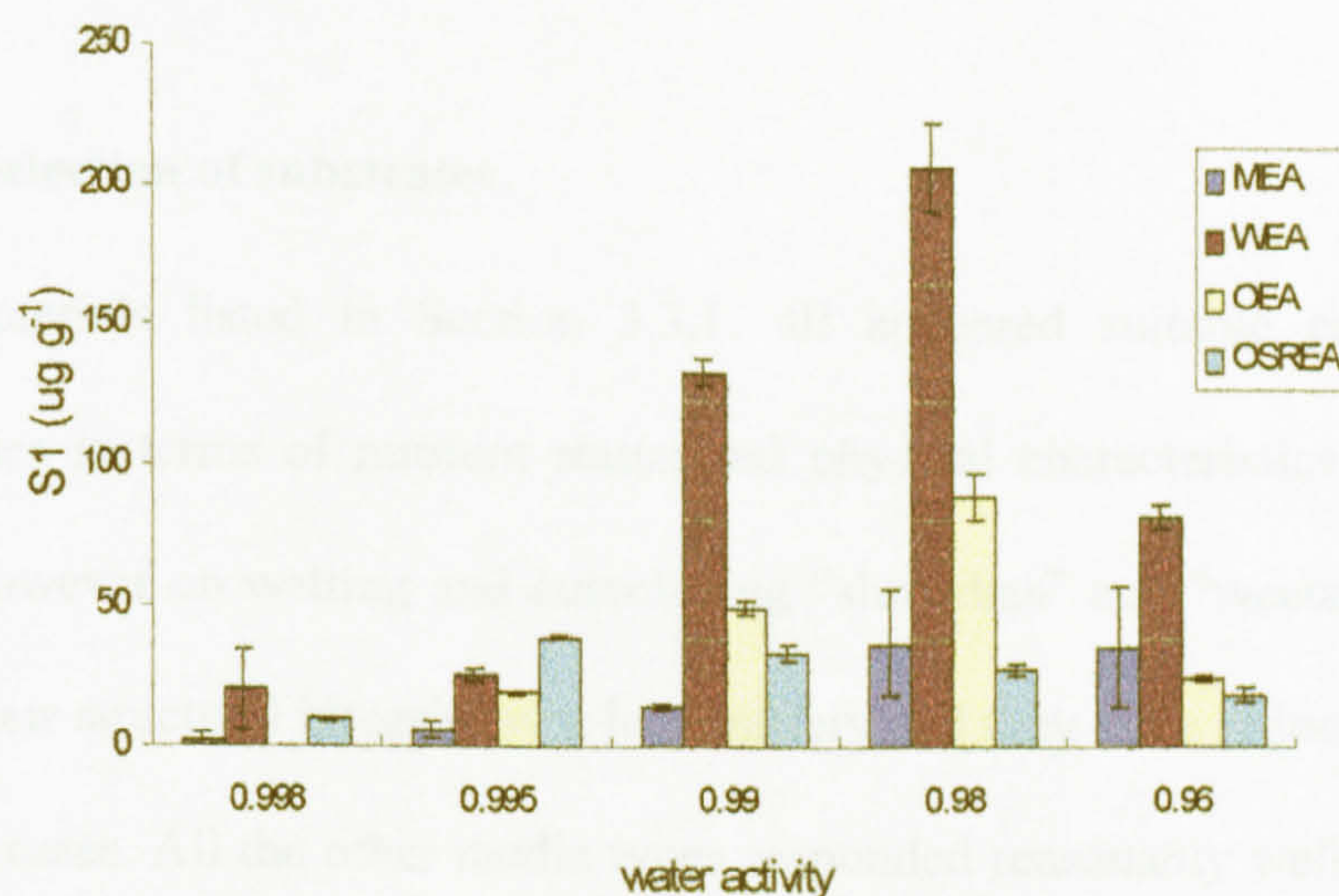


Figure 4.4. (cont.) Influence of medium type and water activity (a_w) on mean yield of squalestatin S1 (expressed as $\mu\text{g g}^{-1}$ wet wt. agar) (mean of three replicates) extracted from colonies of *Phoma* sp. grown for 30 days on malt extract agar (MEA), wheat extract agar (WEA), oat extract agar (OEA) and oil seed rape extract agar (OSREA). Bars are standard error of mean.

4.2. SELECTION OF SUBSTRATES AND MOISTURE SORPTION ISOTHERMS.

4.2.1. Selection of substrates.

The materials listed in Section 3.3.1. all appeared suitable candidates as solid substrates in terms of nutrient status and physical characteristics including particle size. However on wetting and autoclaving “shreddies” and “weetabix” were rejected since their structural integrity was lost entirely and they were reduced to a dense paste in both cases. All the other media types responded reasonably well to autoclaving. In particular, bulgar wheat appeared to retain its structural integrity on repeated autoclaving cycles without any tendency towards particle self-adhesion. Bulgar wheat also exhibited a highly abraded surface texture, presumably due to mechanical pre-treatment (“cracking”) (see Plate 4.2). Couscous was similar but with a slightly greater tendency for “stickiness”. On wetting, wheat flakes produced a generally “fluffy” “open” matrix with a tendency to pack flat in some regions due to the shape of the particles. At the highest a_w level (0.998) most of the substrates appeared obviously wet and substrates such as bulgar wheat and couscous were subject to large volume increases (approx. 100%). At the lowest a_w level (0.96/0.97) most of the substrates appeared comparatively dry with only a limited increase in volume. Millet addition to substrates tended to produce a slightly more dense, caked mass. Millet was therefore used as a supplementary phase in some experiments, it was not used as a sole substrate. Of the whole grains and seeds, wheat, oats and oil seed rape retained their



Plate 4.2. Bulgar wheat, x20 magnification. A – 0.998 a_w , note surface free water. B – 0.97 a_w , note highly abraded surface texture.

integrity well but maize, soya and rice were more difficult to work with. Soya in particular softened markedly and became very fragile, and rice was very “sticky”.

4.2.2. Moisture sorption isotherms.

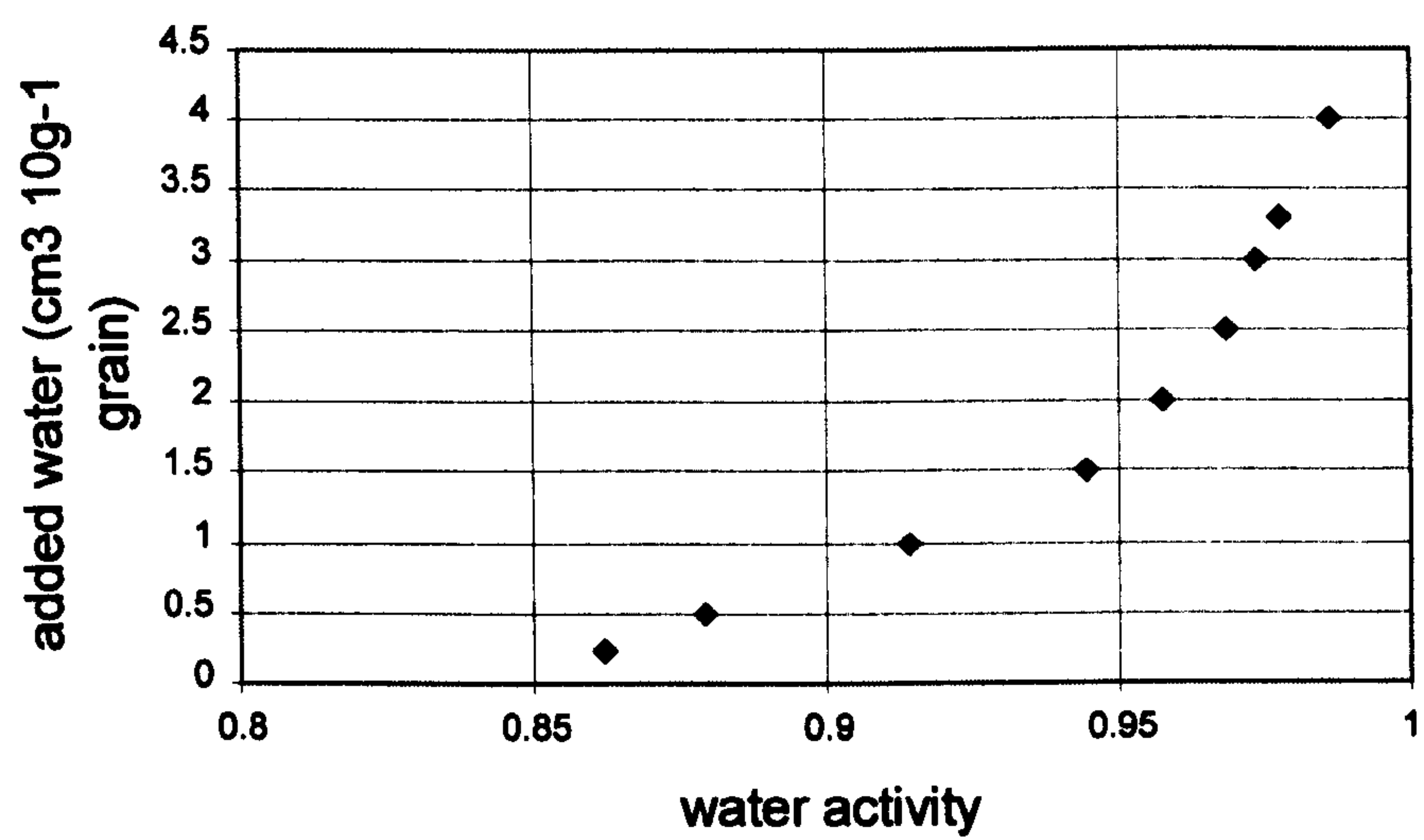
Moisture sorption isotherms were produced for all substrates except for perlite (see Section 5.2.). Figures 4.5 – 4.8. show isotherms for the successfully autoclaved substrates expressed as amount of added water against a_w , and/or percentage moisture content versus a_w . Plots of added water against a_w were included for some substrates since these can be used directly for preparing the substrates at the required a_w levels in subsequent work. However, with a knowledge of the initial water content of the material, plots of % moisture content against a_w are also straightforward to use, so for most substrates this type of plot only was prepared.

Generally, the plots produced show the “wet end” of the classic moisture sorption isotherm curve, consisting of an initial “flat” region where small additions of water cause large changes in a_w . The curve then becomes steeper at the highest water content levels, as the substrate begins to reach saturation. The moisture sorption isotherms produced were remarkably uniform across the range of substrate types investigated. For example, the “raw materials” as a group (i.e. wheat, oats, oil seed rape, maize, rice, soya) all produced similar results, with 0.90 a_w falling in the range 15 – 20% moisture, 0.95 a_w in the range 20 – 25% moisture, and saturation (approx. 1.00 a_w) at around 40% moisture. The “processed” materials (wheat flakes, bulgar wheat, couscous, shredded wheat, medium A) all appeared to fall into a second broad

group with typically 0.95 a_w at approx. 30% moisture and saturation in the range 50 – 70% moisture.

From the partial isotherm constructed for wheat flakes plus millet there was little evidence that millet addition significantly altered the wheat flakes isotherm. Therefore no additional isotherm was constructed for the other mixed substrate used in this study (couscous plus millet). It was assumed that the couscous isotherm could be used directly.

a. Relationship between added water and a_w .



b. Relationship between % water content (wet wt. basis) and a_w .

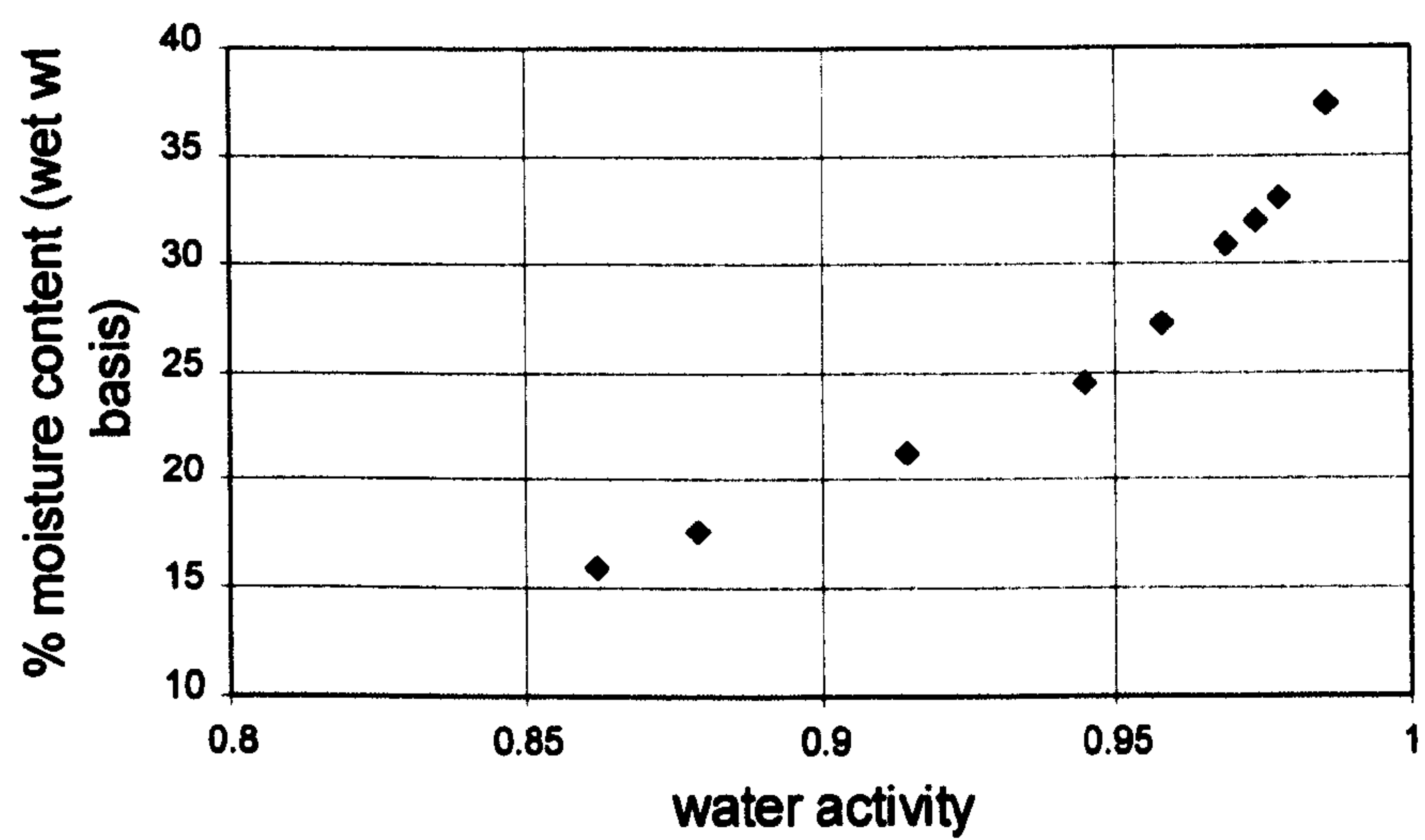
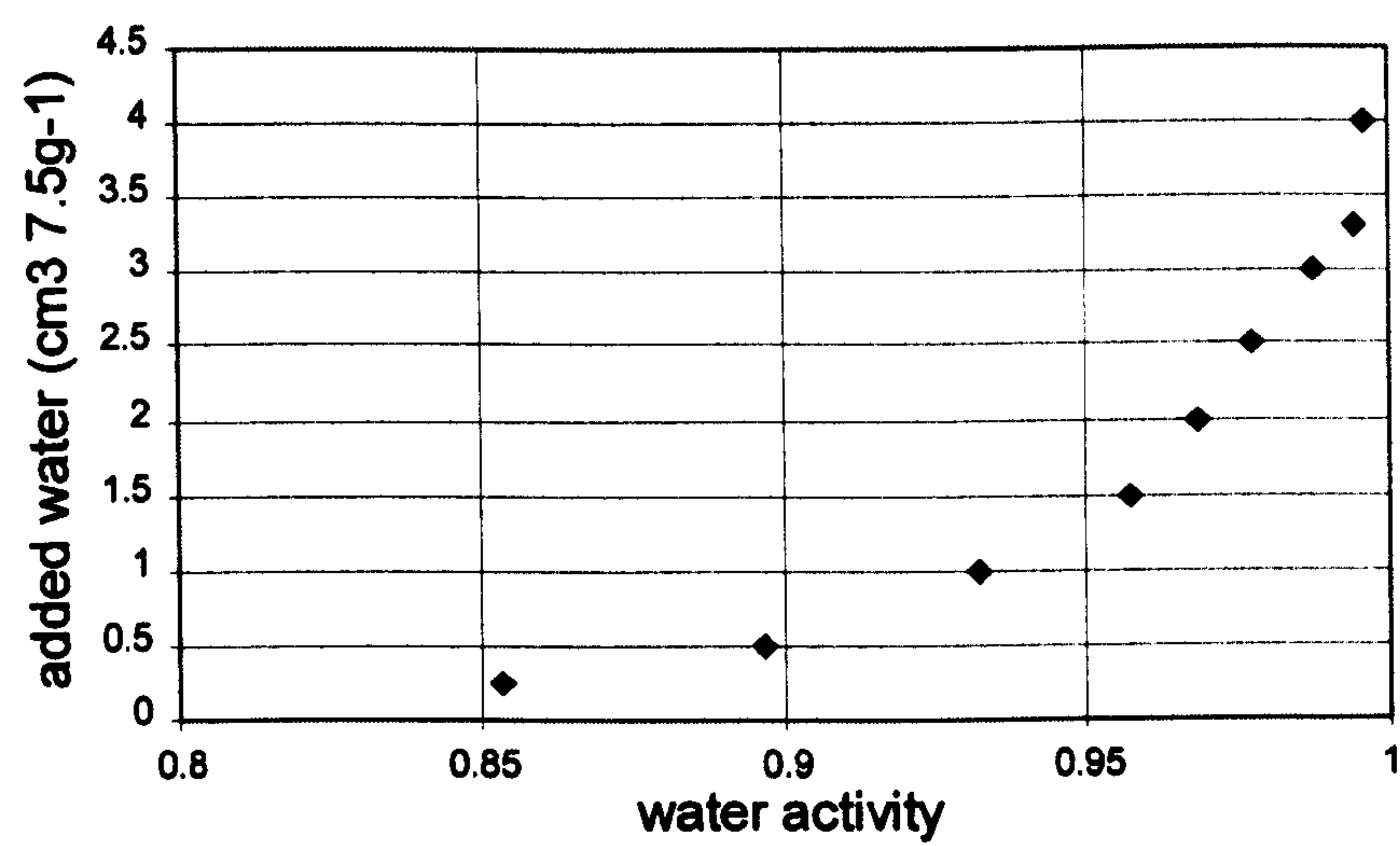


Figure 4.5. Moisture sorption isotherms for whole autoclaved wheat grain at 25°C.

a. Relationship between added water and a_w .



b. Relationship between % water content (wet wt. basis) and a_w .

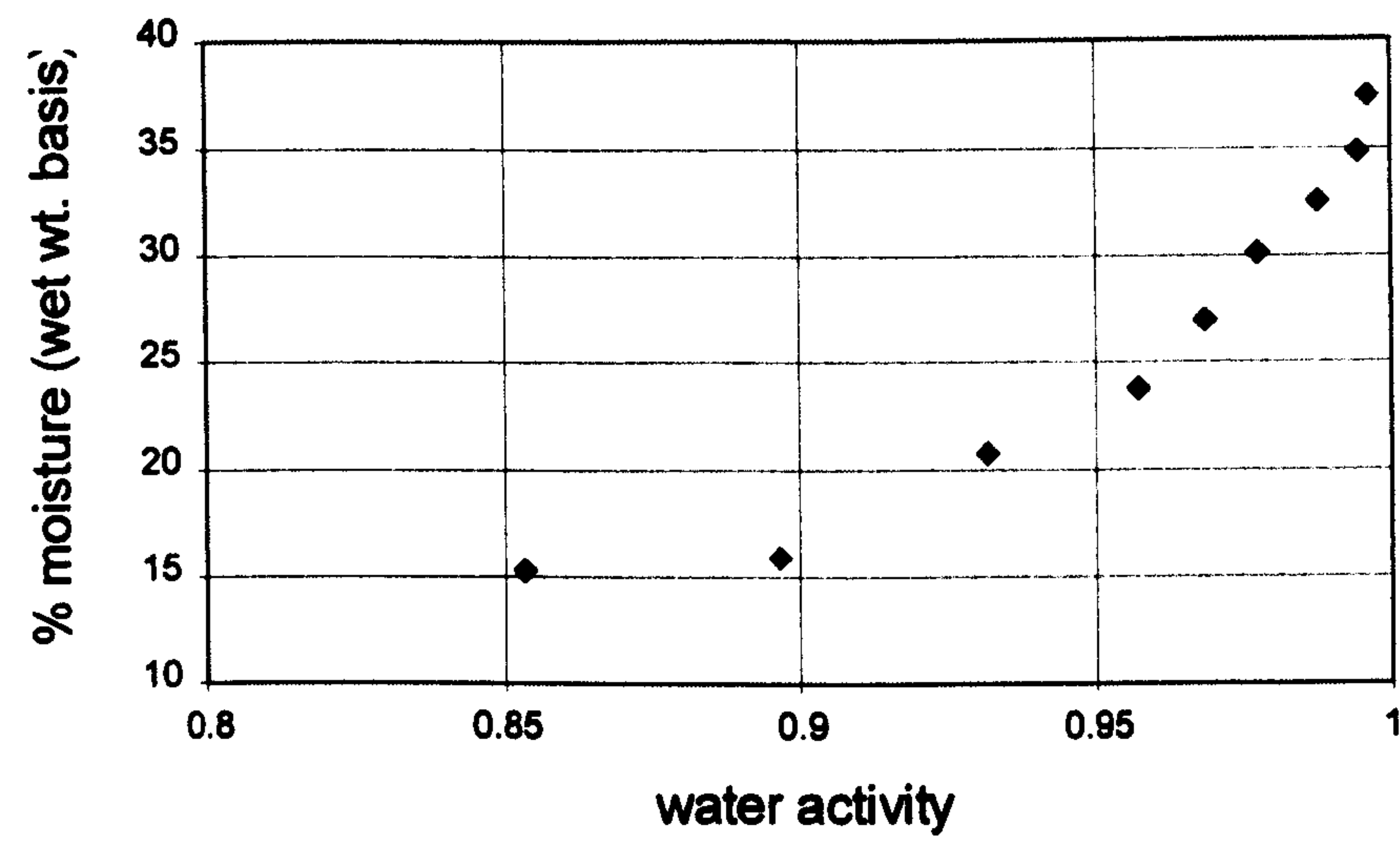
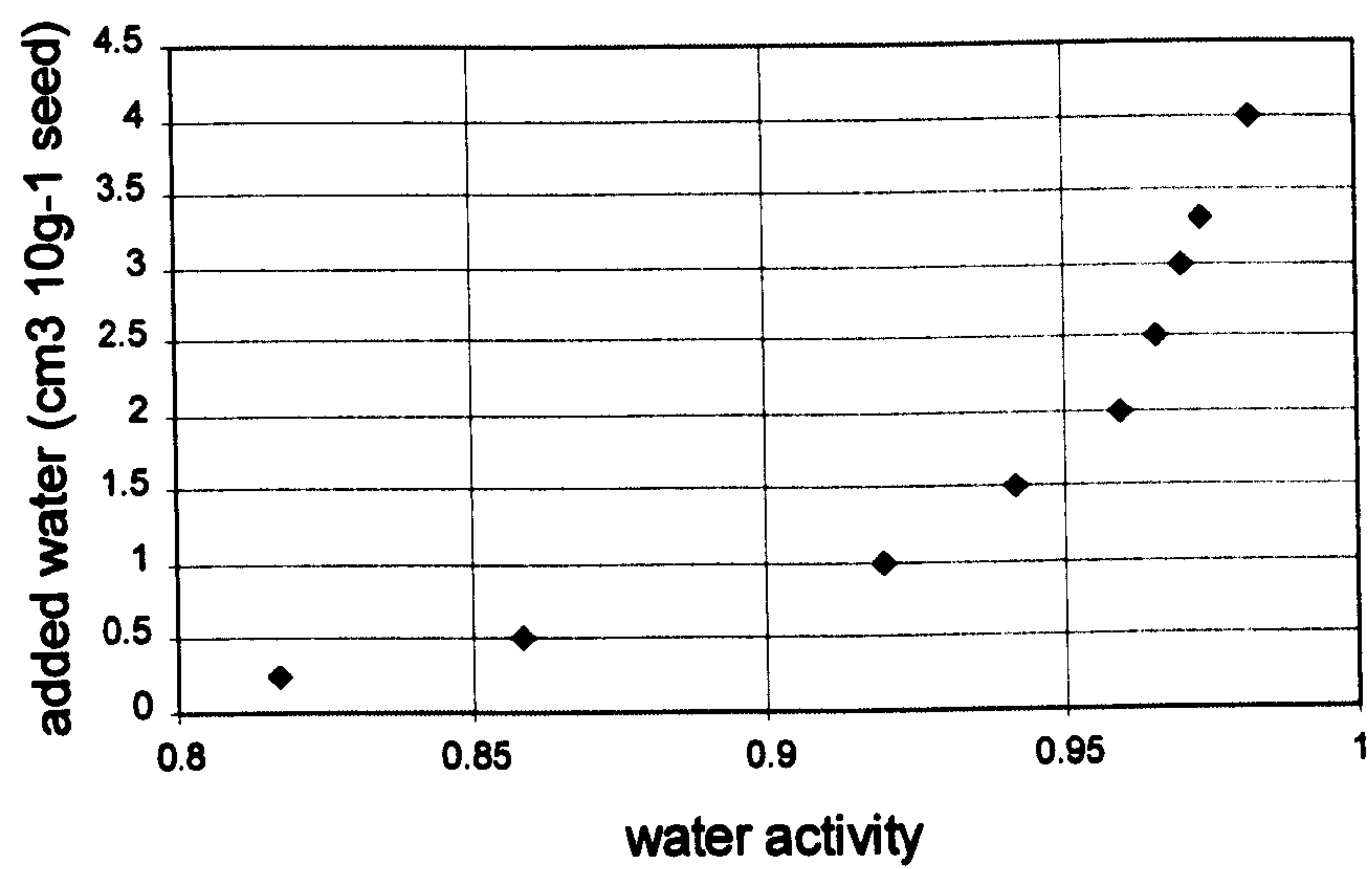


Figure 4.6. Moisture sorption isotherms for whole autoclaved oat grain at 25°C.

a. Relationship between added water and a_w .



b. Relationship between % water content (wet wt. basis) and a_w .

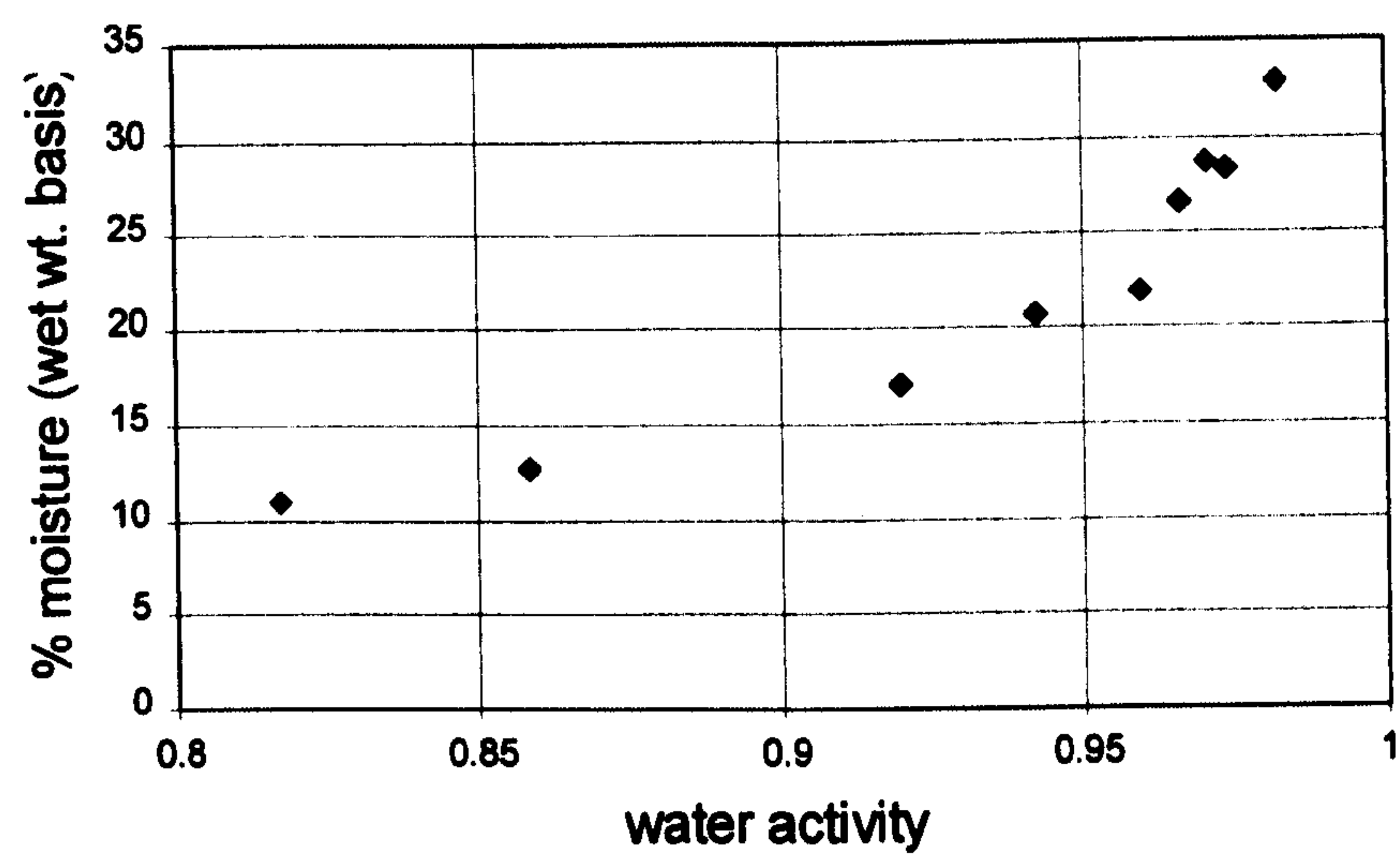
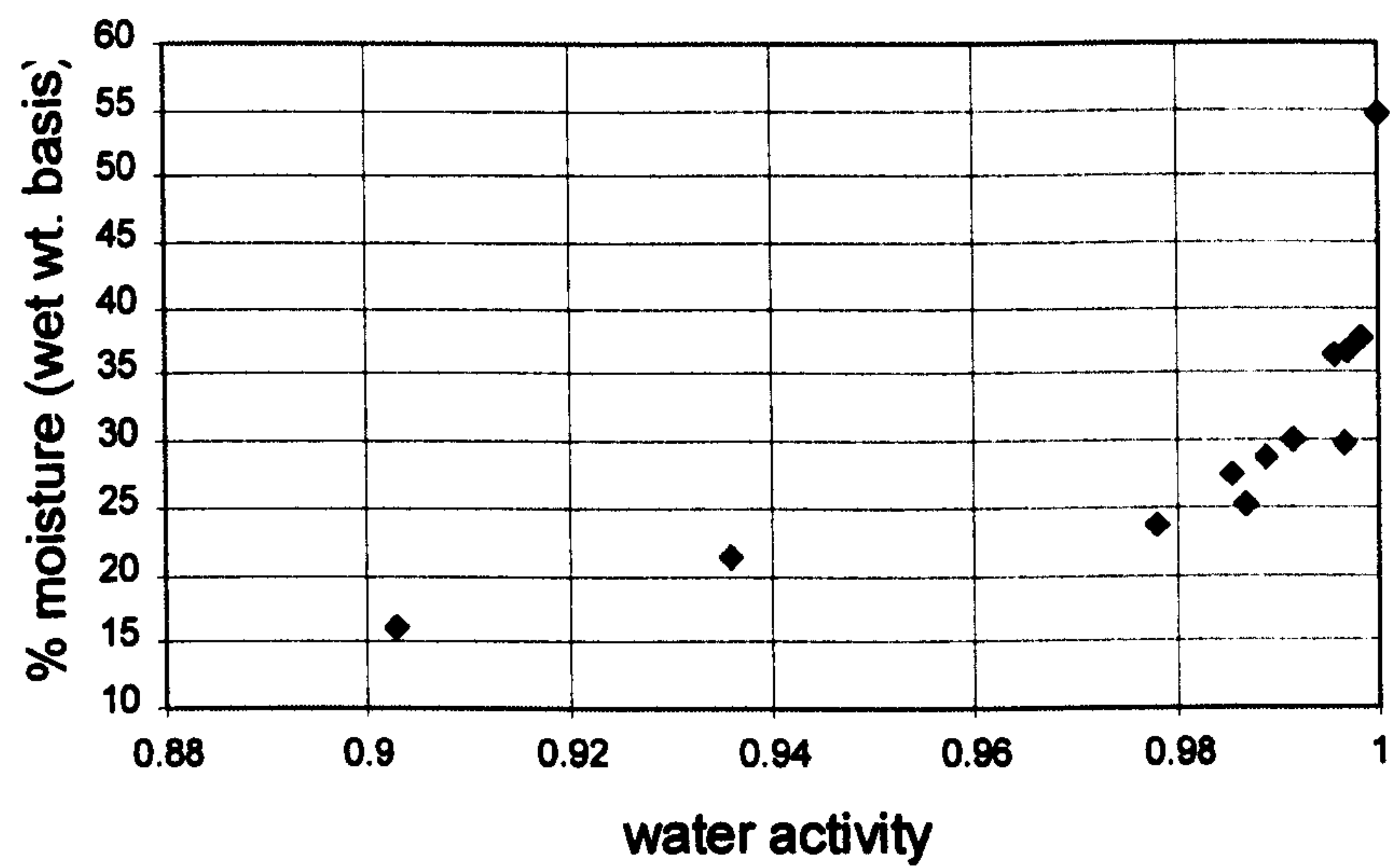


Figure 4.7. Moisture sorption isotherms for whole autoclaved oil seed rape at 25°C.

a. Whole Spanish maize.



b) whole soya beans.

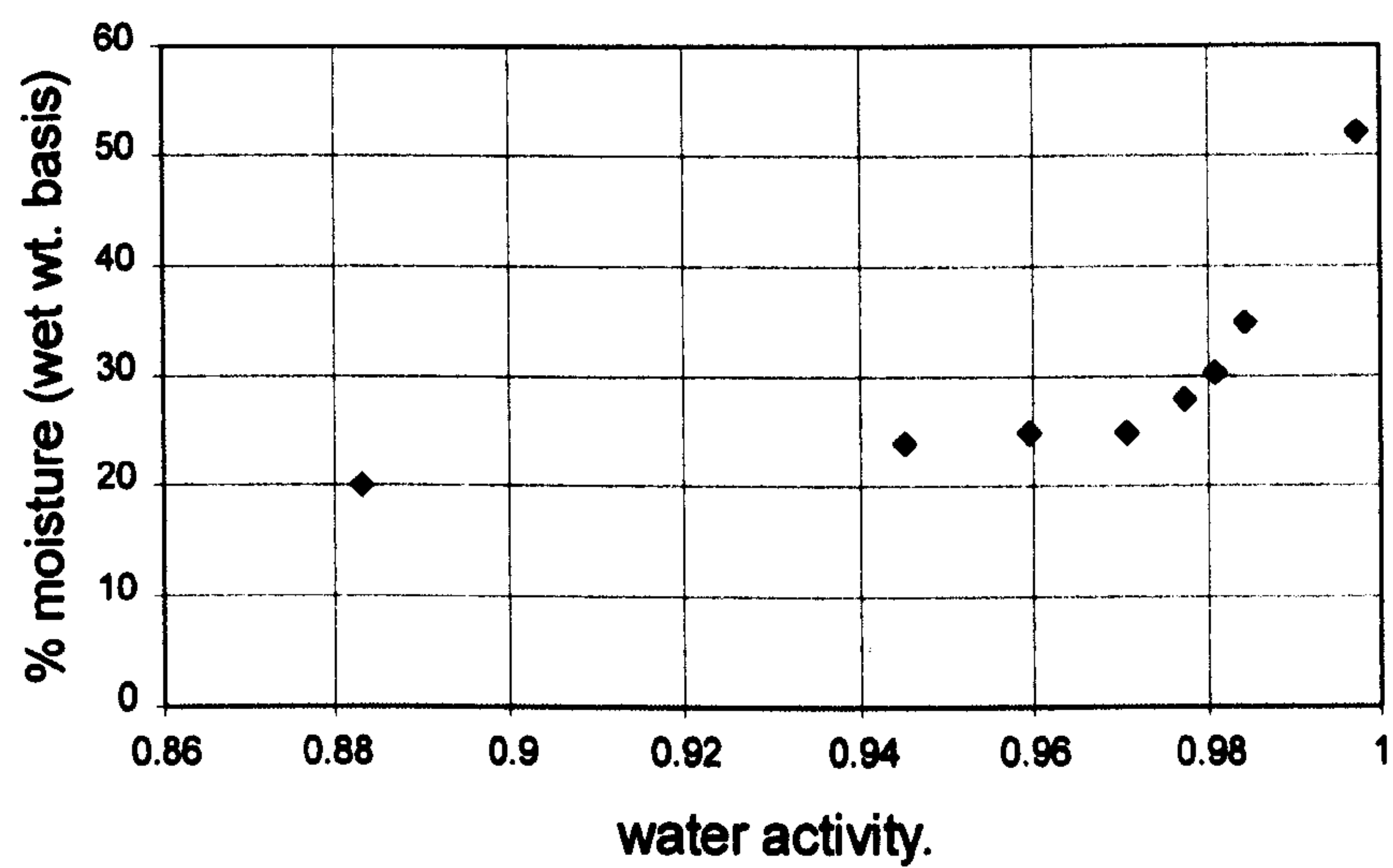
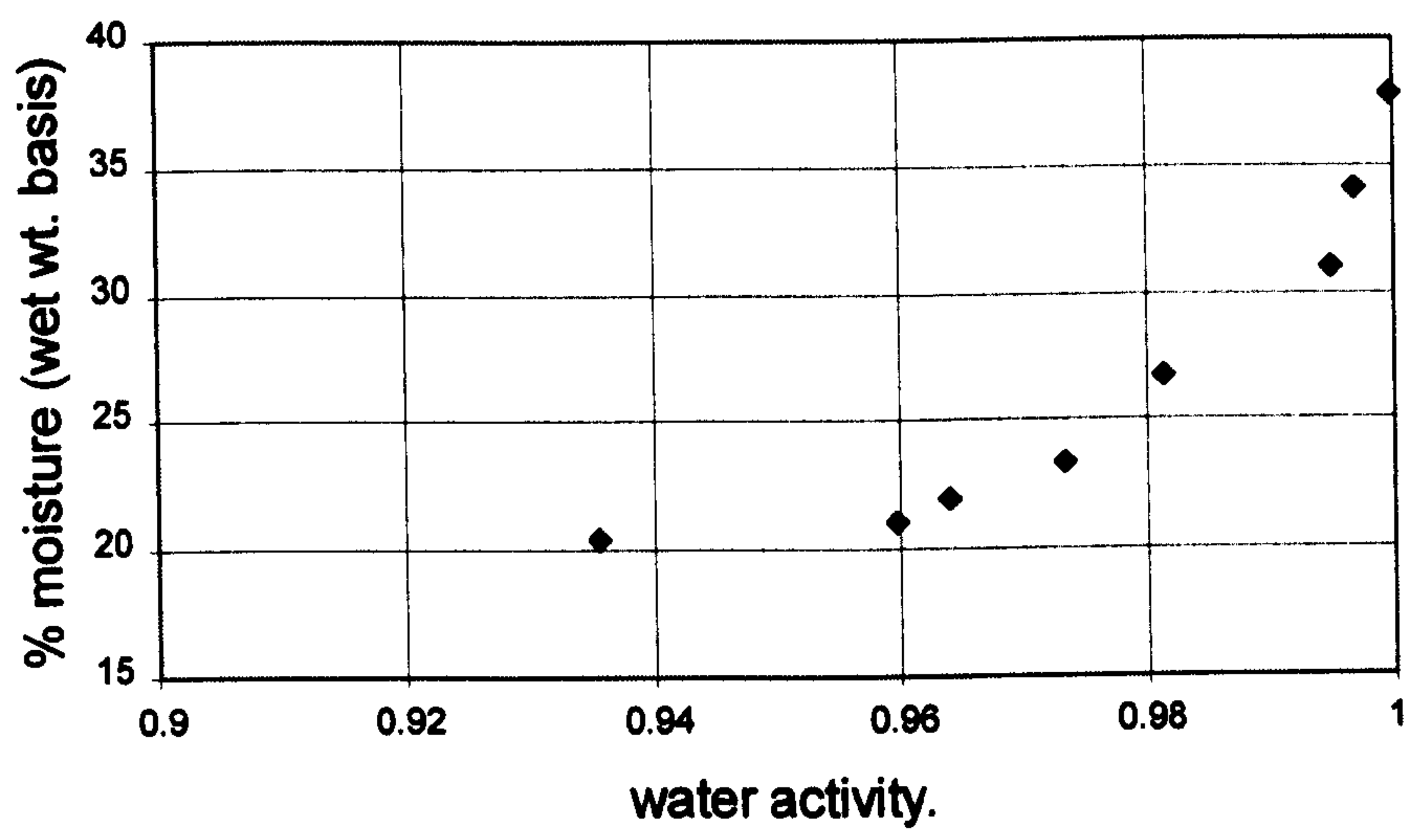


Figure 4.8. Moisture sorption isotherms for whole autoclaved substrates at 25°C.

Relationship between % moisture content (wet wt. basis) and a_w .

c. American long-grain rice.



d. “Shredded wheat”.

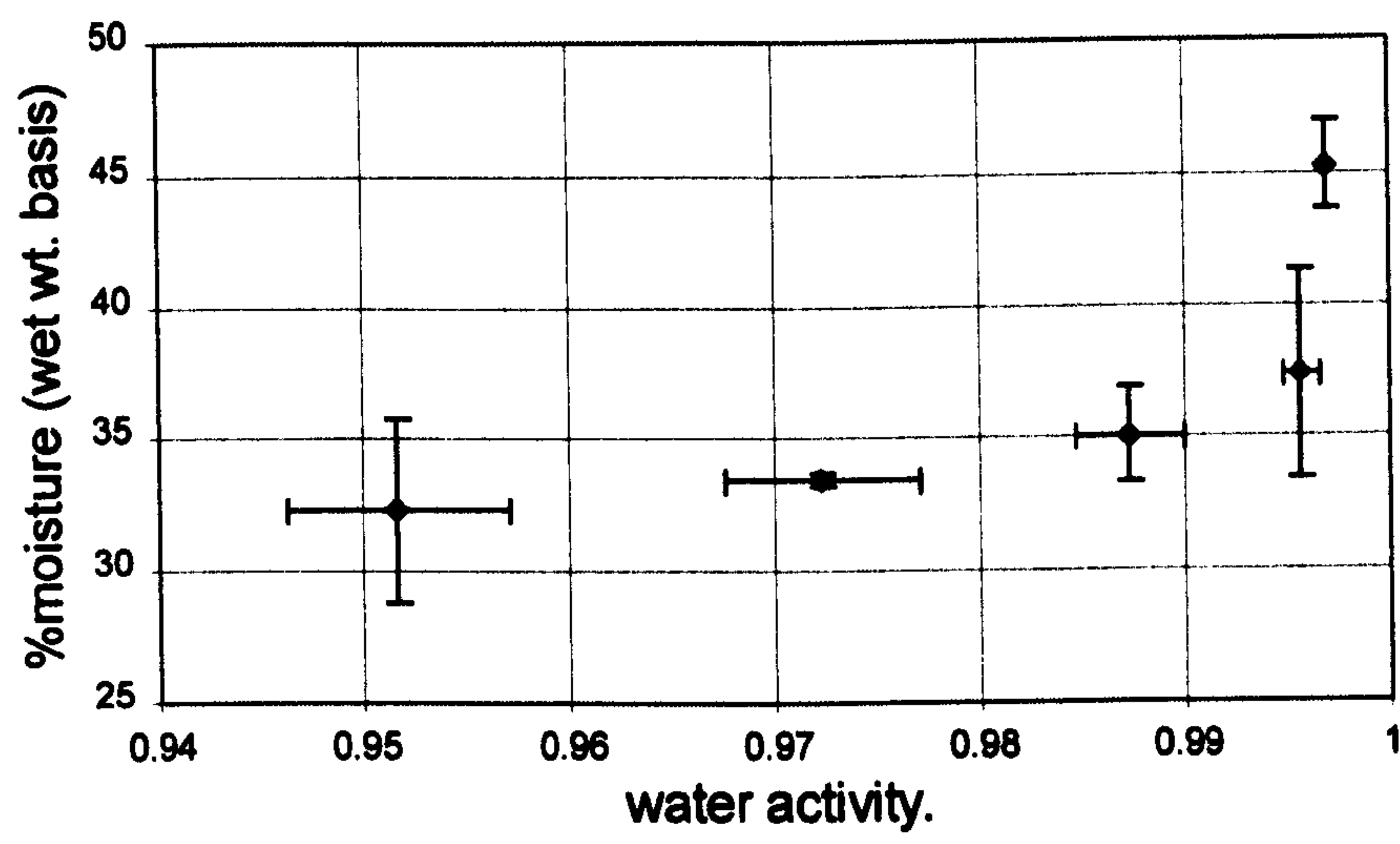
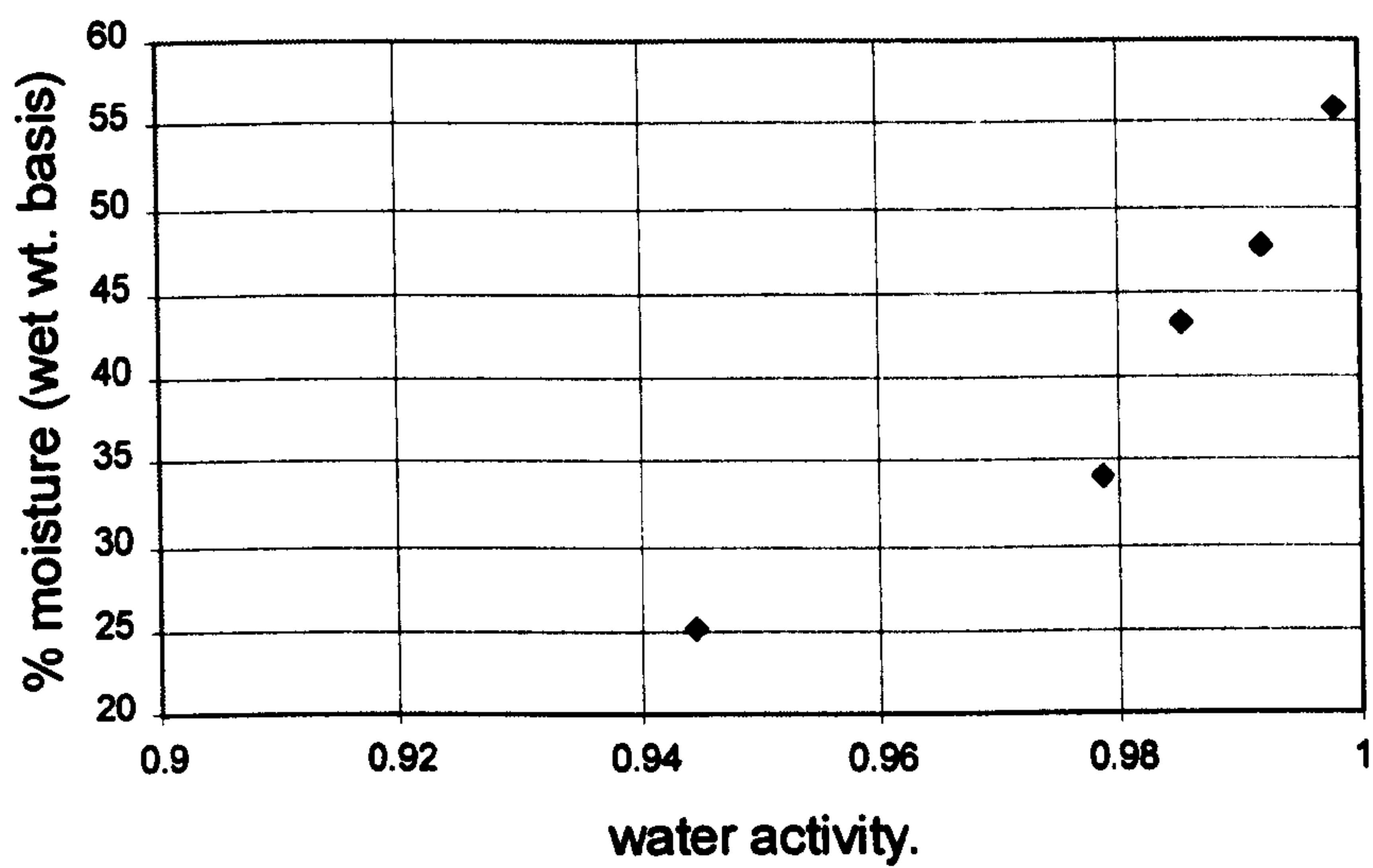


Figure 4.8. (cont.) Moisture sorption isotherms for whole autoclaved substrates at 25°C. Relationship between % moisture content (wet wt. basis) and a_w . Error bars on (d) are standard errors of mean.

e. Wheat flakes.



f. Bulgar wheat

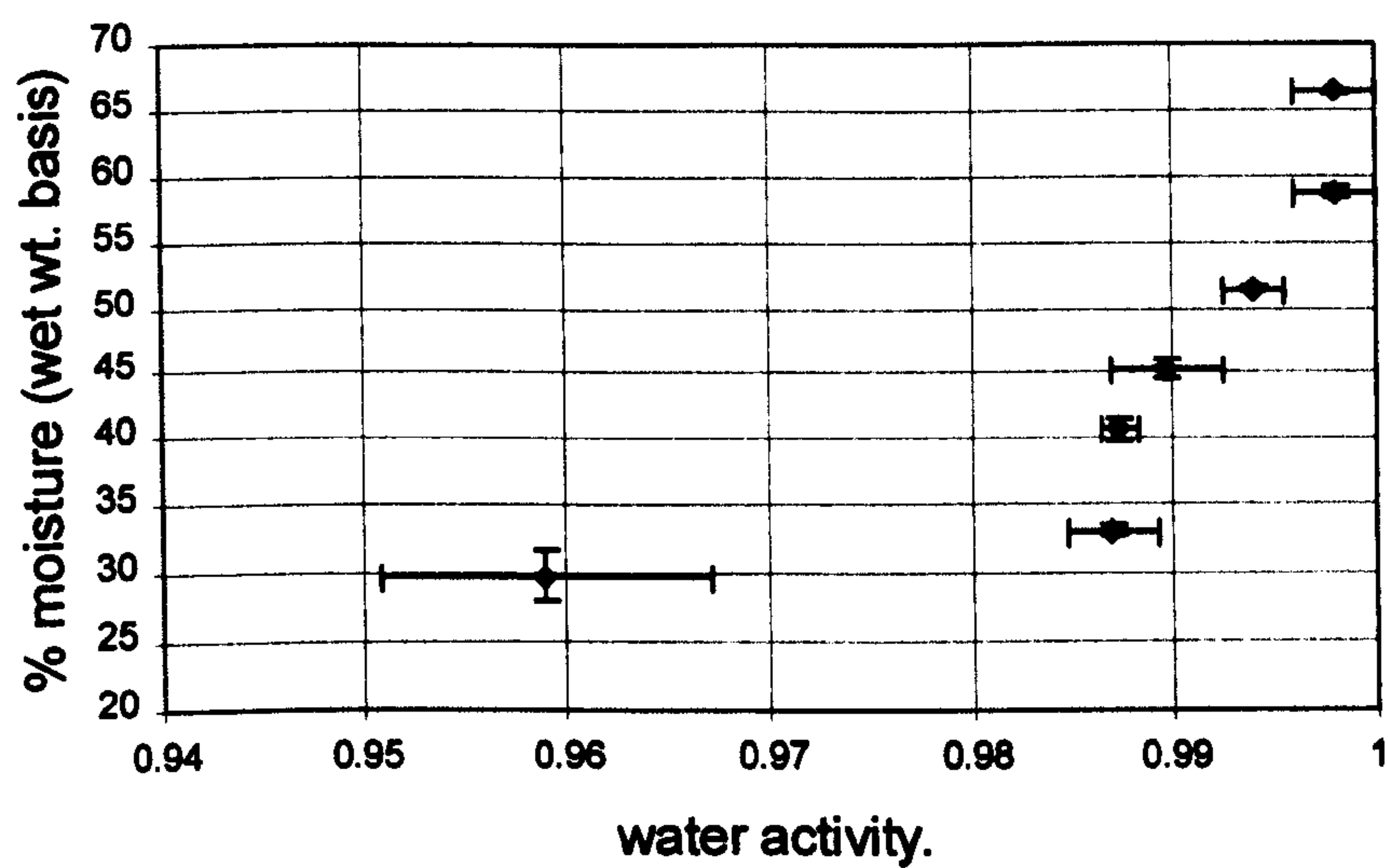
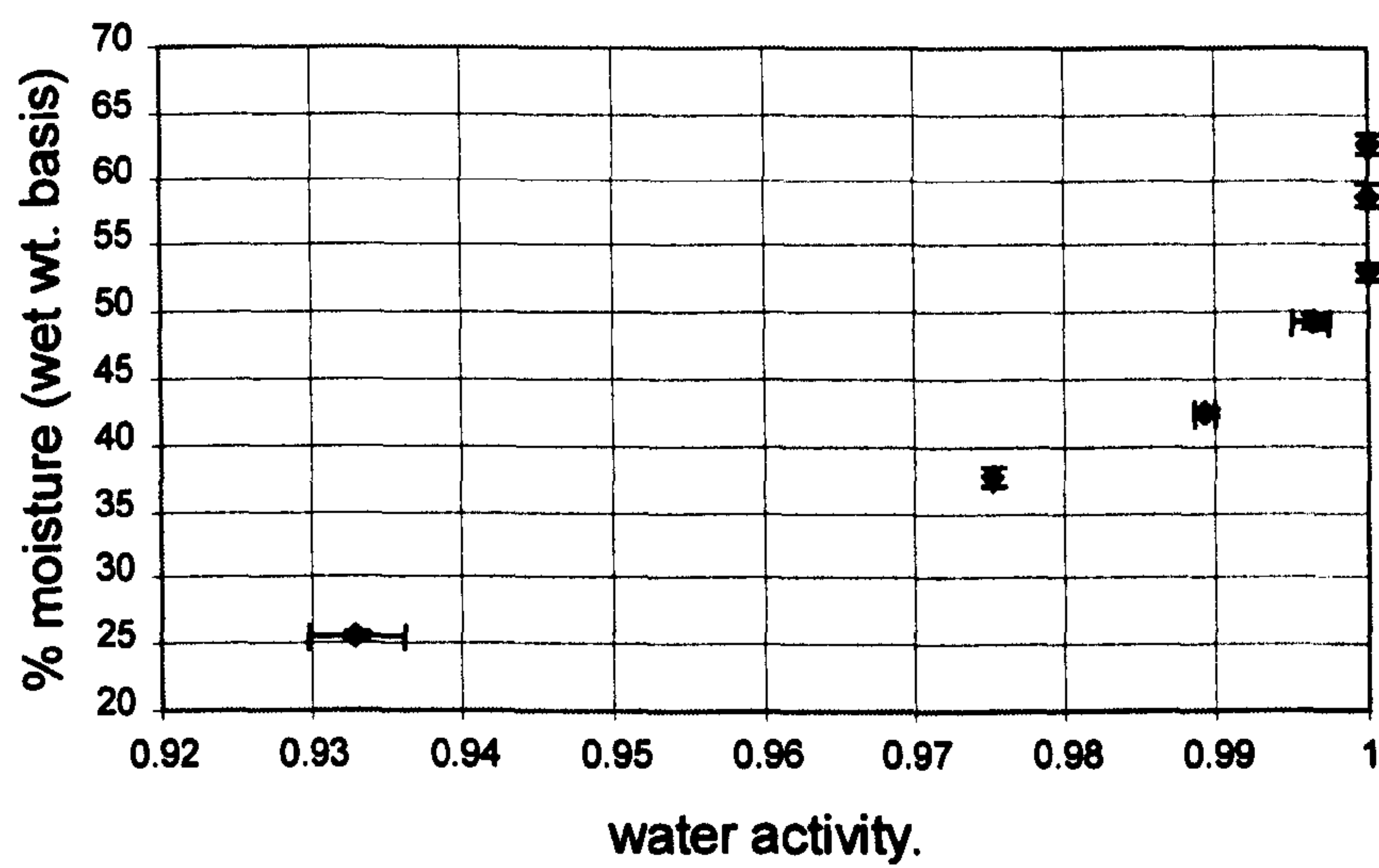


Figure 4.8. (cont.) Moisture sorption isotherms for whole autoclaved substrates at 25°C. Relationship between % moisture content (wet wt. basis) and a_w . Error bars on (f) are standard errors of mean.

g. Couscous



h. Flaked wheat +flaked millet.

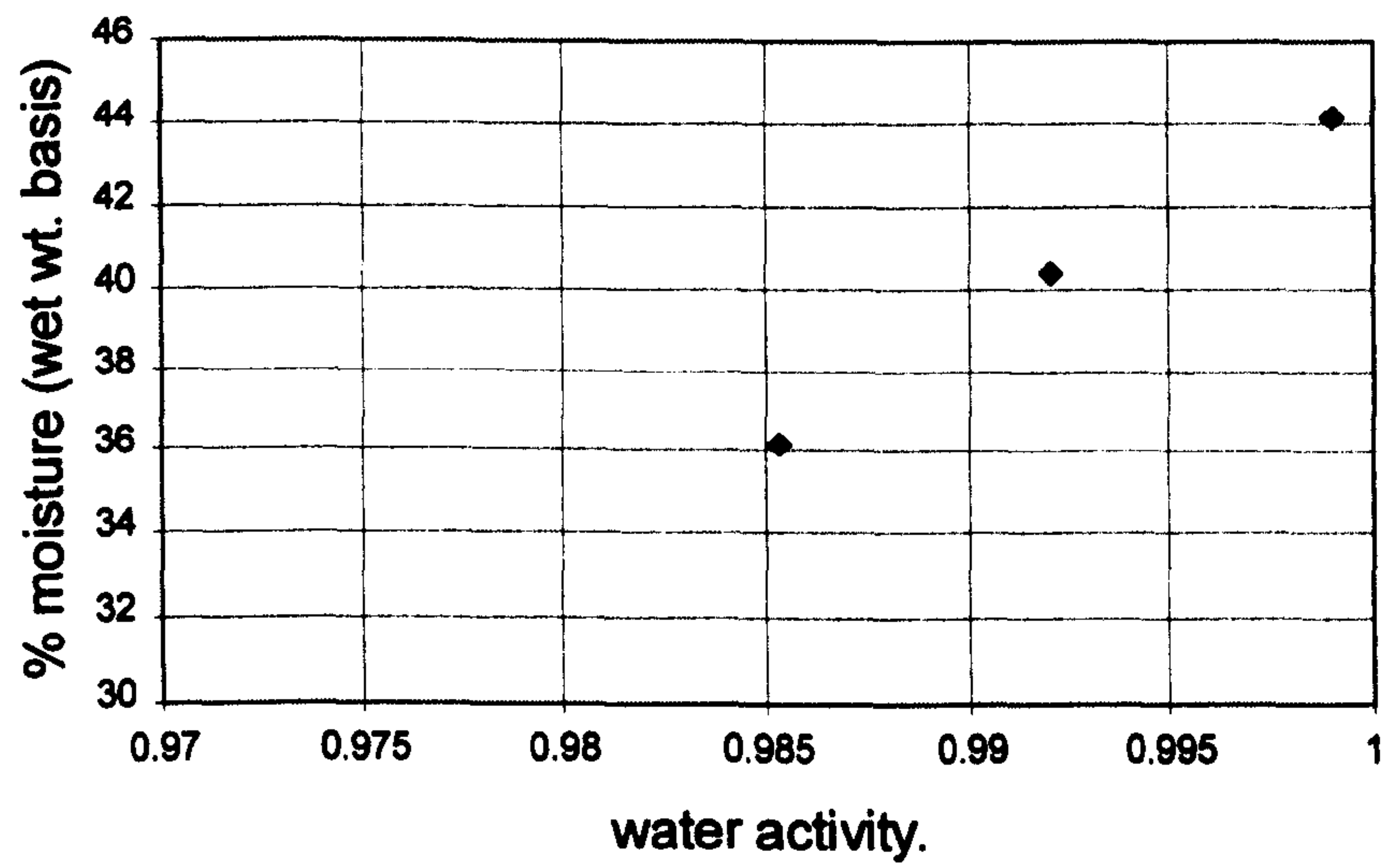


Figure 4.8. (cont.) Moisture sorption isotherms for whole autoclaved substrates at 25°C. Relationship between % moisture content (wet wt. basis) and a_w . Error bars on (g) are standard errors of mean.

i. Terragen medium A.

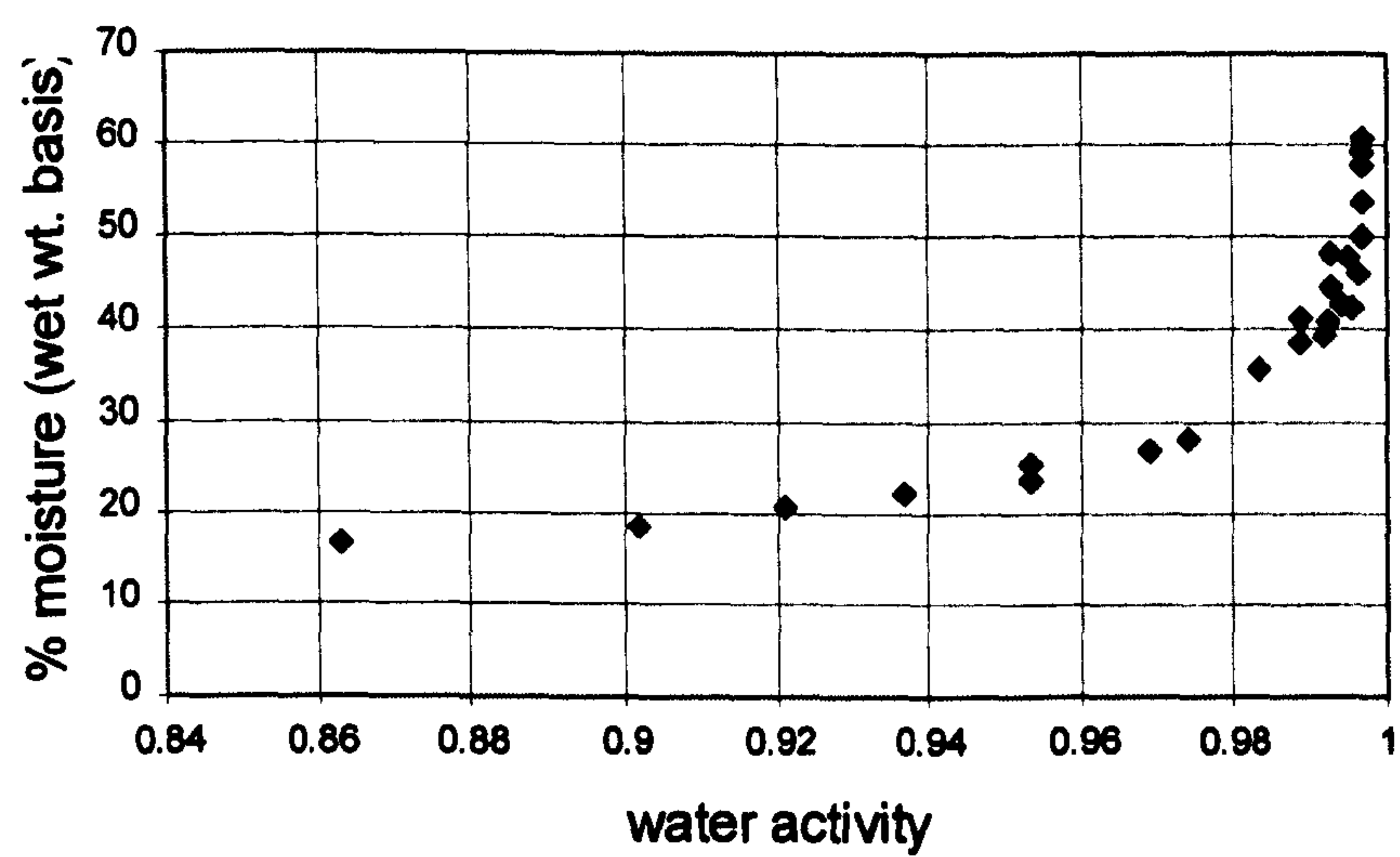


Figure 4.8. (cont.) Moisture sorption isotherms for whole autoclaved substrates at 25°C. Relationship between % moisture content (wet wt. basis) and a_w

4.3. GROWTH AND SECONDARY METABOLITE PRODUCTION ON SOLID SUBSTRATES.

4.3.1. Growth of fungal species on solid substrates.

1. Corynespora cassiicola.

C. cassiicola was grown on wheat, oats, oil seed rape, maize, rice, soya beans and TerraGen medium A. All treatments contained an inert second phase + supplements (See Plate 4.3 for arrangement of two-phase substrate treatments). For all media, growth was surprisingly uniform across the range of a_w levels. 100% mycelial cover was recorded in each case, becoming slightly more diffuse as a_w declined. A pink pigment was evident, being localised mainly at the interface of the two substrate phases for all a_w levels except 0.97, where the pigmentation was more generally distributed over the mycelium. Sporulation was absent from all treatments.

2. Drechslera dematioidea

D. dematioidea was grown on wheat, oats, oil seed rape and TerraGen medium A (all treatments contained an inert second phase + supplements). 100% mycelial growth was recorded for all medium types at 0.998 a_w . This growth was dense for wheat and medium A, and more diffuse for oats and oil seed rape. In addition there was approx. 50% spore cover for medium A, oats and oil seed rape, and 75% cover for wheat. As a_w declined mycelial growth remained unchanged but sporulation increased to 100% cover for all treatments.



Plate 4.3. Set-up of two phase treatments, in this case oats and perlite.

3. *Sarophorum palmicola*.

S. palmicola was grown on wheat, oats, oil seed rape, maize, rice, soya beans and TerraGen medium A (all treatments contained an inert second phase + supplements). *S. palmicola* typically produces striking phototrophic stromata (mitospore forming structures) on a sparse basal mycelium. There was copious development of these structures for wheat, soya, maize and medium A at 0.997 a_w (see Plate 4.4), and reduced stroma production in oats, rice and oil seed rape at this a_w level. *S. palmicola* responded very strongly to lowered a_w . For all media types growth and stroma production were dramatically reduced as a_w was lowered, to the extent that at 0.970 a_w stroma production was totally absent for oats, oil seed rape and rice, with only traces of reduced stroma for wheat, maize, soya and TerraGen medium A.

4. *Epicoccum nigrum*.

E. nigrum was grown on wheat, oats, oil seed rape, shredded wheat (all with an inert second phase + supplements), wheat flakes, supplemented wheat flakes, wheat flakes + inert phase, wheat flakes + millet, bulgar wheat, couscous, couscous + millet and TerraGen medium A. Generally, growth was extremely uniform for all media types. Typically, there was 100% cover of a dense off-white mycelium at 0.998 a_w which tended to become more diffuse as a_w diminished, but % cover remained unchanged. Exceptions to this were shredded wheat and oil seed rape, where growth was reduced generally and severely inhibited at 0.970 a_w . In particular, there were no discernible differences between growth patterns for the various wheat flake treatments carried out.



Plate 4.4. *Sarophorum palmicola* growing on medium A (2 weeks, 25°C). Note phototrophic stroma).

There was marked production of pigments, predominantly pink/red at high a_w and yellow at low a_w (see Plate 4.5). Of the four organisms tested with these substrates, growth of *E. nigrum* seemed to be least affected by changing a_w level.

4.3.2. Secondary metabolite profiles of fungal spp. on solid substrates.

Presentation of results.

Secondary metabolite profiles were constructed from HPLC data for each organism and substrate type. These profiles are charts of metabolite peak area (measured in absorption units (UV * sec)) versus elution time (measured in minutes). For each metabolite a grouped set of results is shown corresponding to the a_w range employed in the experiment. For each organism the profile for TerraGen medium A is given first followed by profiles for the other media used. Profiles are not always to the same scale but vary to optimise expression of all the metabolites obtained. Retention times used in the profiles are as obtained in the HPLC data and vary slightly between experiments in some cases. For example, for *C. cassicola* growing on TerraGen medium A and wheat, a common metabolite elutes at 4.970 min and 4.945 min respectively (see Fig. 4.9.(a) and (b)). Elution times which vary by less than 0.1 min between experiments are common metabolites (unless otherwise stated). All profiles were corrected against uninoculated substrate control samples.



Plate 4.5. *Epicoccum nigrum* growing on bulgar wheat (2 weeks, 25°C). Clockwise from top left: 0.998, 0.990, 0.970, 0.980 a_w . Note red pigment at high a_w and yellow pigment at lower a_w .

In addition to the secondary metabolite profiles, comparative charts were constructed for each organism showing variation in target metabolites over the range of substrates employed. The metabolite levels shown in these charts are the maximum levels achieved for each substrate and do not show a_w variation. The charts also incorporate reference levels of each target peak originally obtained in previous work carried out at TerraGen Discovery Ltd.

1. *C. cassiicola*.

(a) Effects of medium type on secondary metabolite profiles.

Secondary metabolite profiles for *C. cassiicola* are shown in Figs 4.9. (a) – (f). The profiles show the complete range of peaks obtained for this organism. The profiles are strikingly different, in terms of metabolite range and levels, in contrast to the relatively uniform growth responses obtained for the range of substrates. OSR and soya produced the widest range of metabolites, and it may be significant that these two media both have a relatively high oil content. The most limited metabolite ranges were produced by wheat, rice and maize. Maize culture in fact produced no metabolites under the conditions employed. There was frequently a high standard error for a given metabolite result, and this was often due to only one or two of the three replicates giving a result.

(b) Effects of a_w on secondary metabolite profiles.

In general, *C. cassiicola* did not respond particularly strongly to changes in a_w level. Results either remained reasonably uniform or declined slightly as a_w was lowered.

In addition to the secondary metabolite profiles, comparative charts were constructed for each organism showing variation in target metabolites over the range of substrates employed. The metabolite levels shown in these charts are the maximum levels achieved for each substrate and do not show a_w variation. The charts also incorporate reference levels of each target peak originally obtained in previous work carried out at TerraGen Discovery Ltd.

1. *C. cassiicola*.

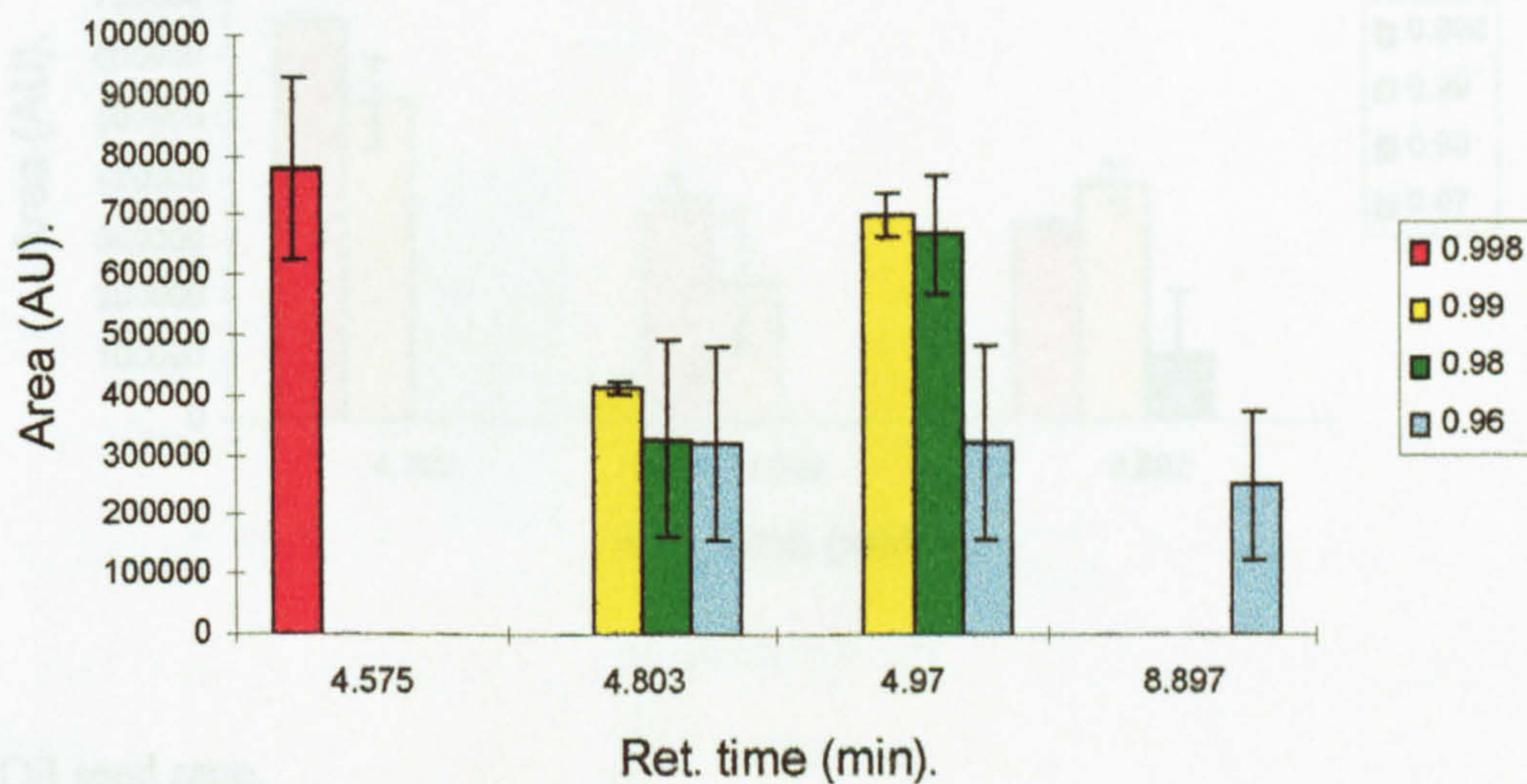
(a) Effects of medium type on secondary metabolite profiles.

Secondary metabolite profiles for *C. cassiicola* are shown in Figs 4.9. (a) – (f). The profiles show the complete range of peaks obtained for this organism. The profiles are strikingly different, in terms of metabolite range and levels, in contrast to the relatively uniform growth responses obtained for the range of substrates. OSR and soya produced the widest range of metabolites, and it may be significant that these two media both have a relatively high oil content. The most limited metabolite ranges were produced by wheat, rice and maize. Maize culture in fact produced no metabolites under the conditions employed. There was frequently a high standard error for a given metabolite result, and this was often due to only one or two of the three replicates giving a result.

(b) Effects of a_w on secondary metabolite profiles.

In general, *C. cassiicola* did not respond particularly strongly to changes in a_w level. Results either remained reasonably uniform or declined slightly as a_w was lowered.

a. Medium A.



b. Wheat.

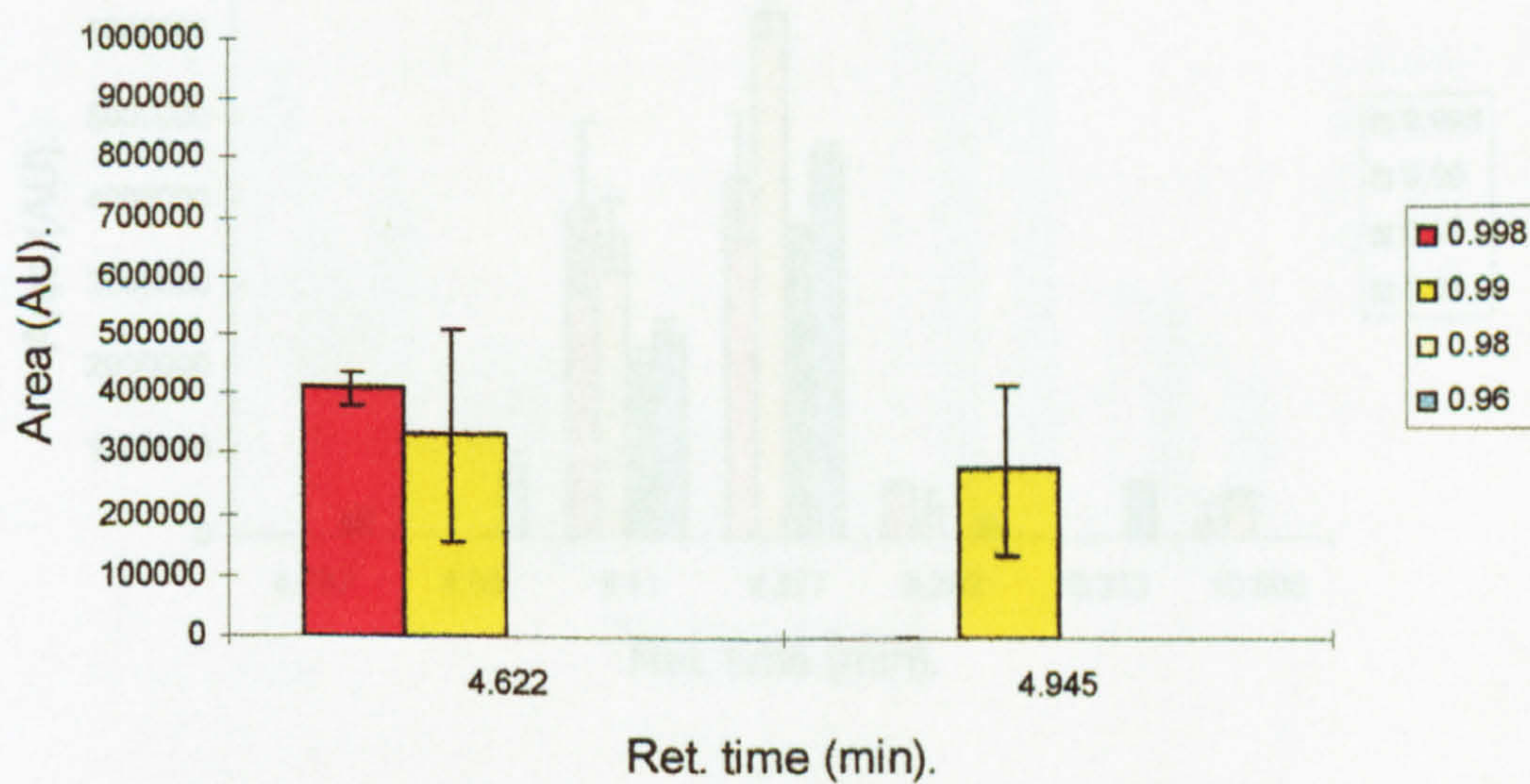
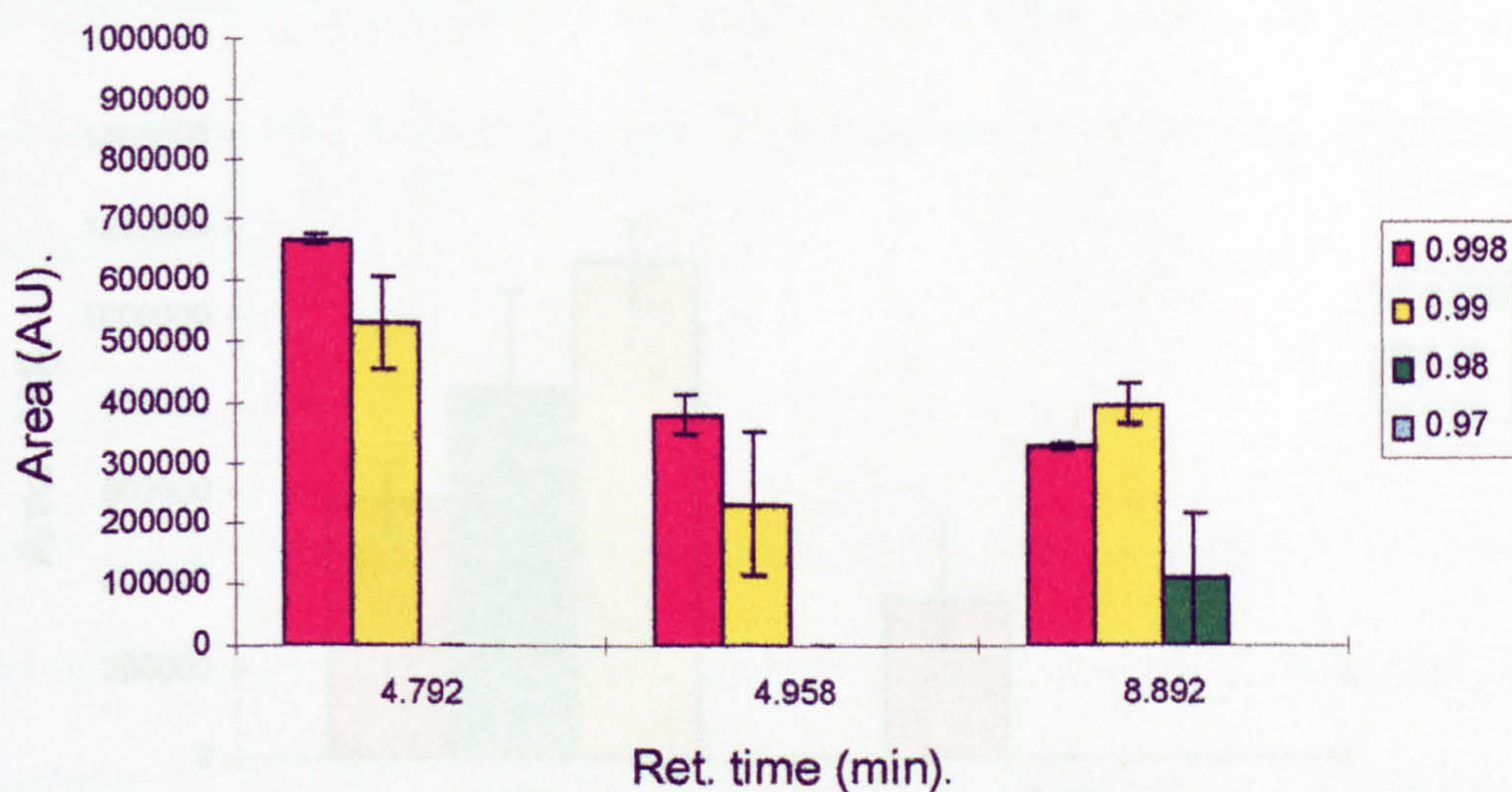


Figure 4.9. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *C. cassiicola*. Bars are standard error of mean. Legend shows a_w range

c. Oats.



d. Oil seed rape.

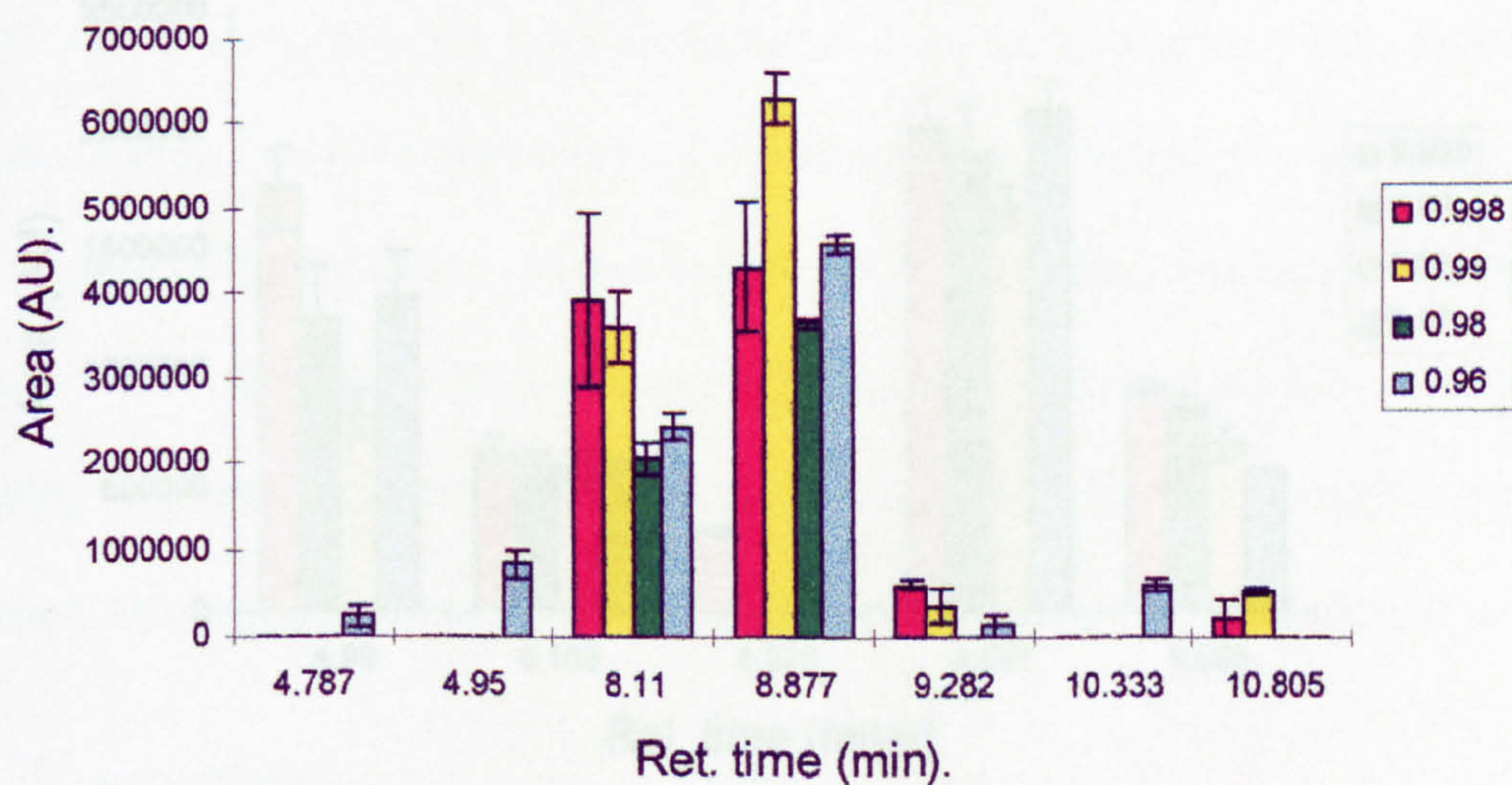
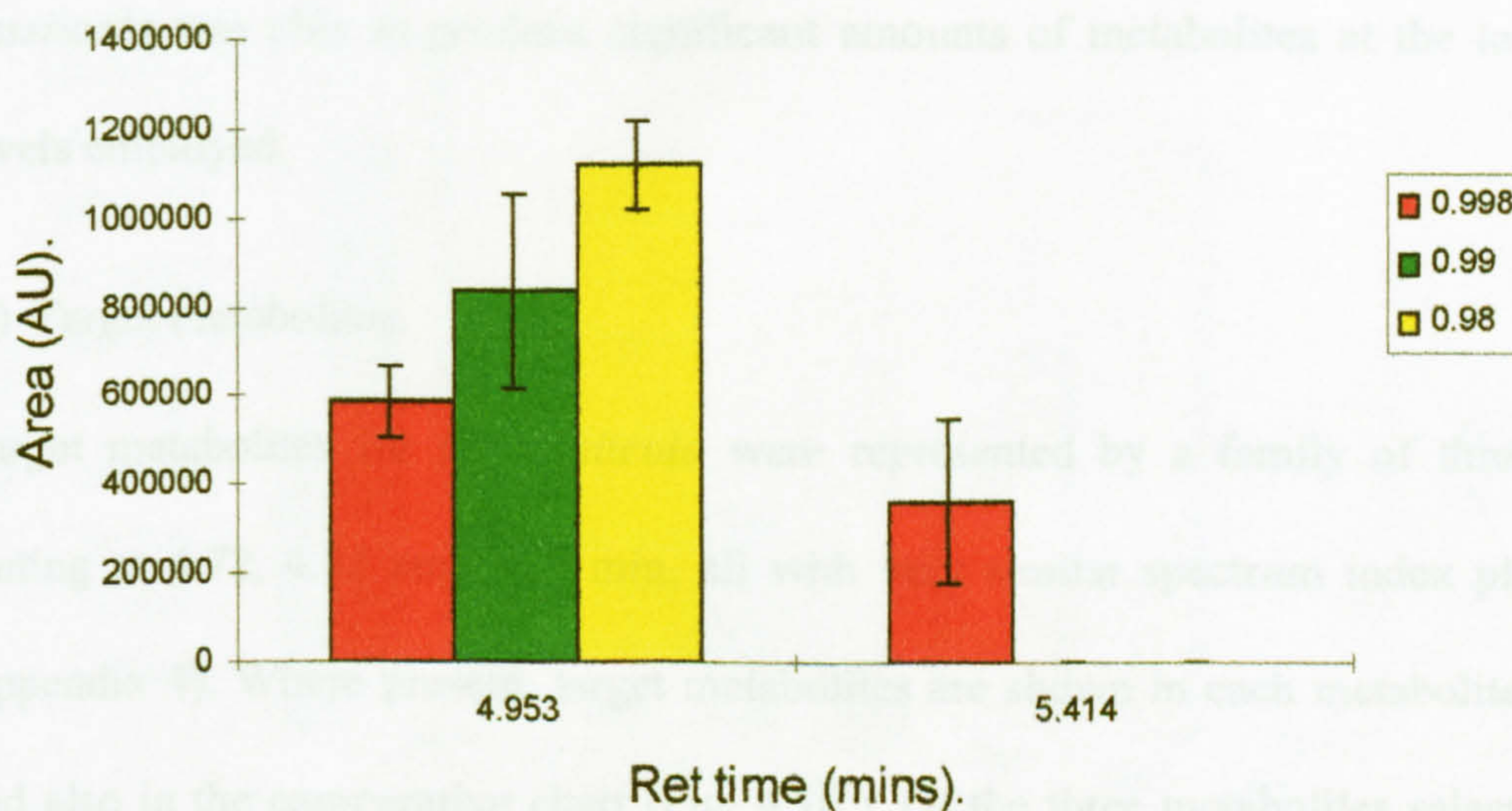


Figure 4.9. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs

Figure 4.9. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *C. cassicola*. Bars are standard error of mean. Legend shows a_w range.

e. Rice.



f. Soya.

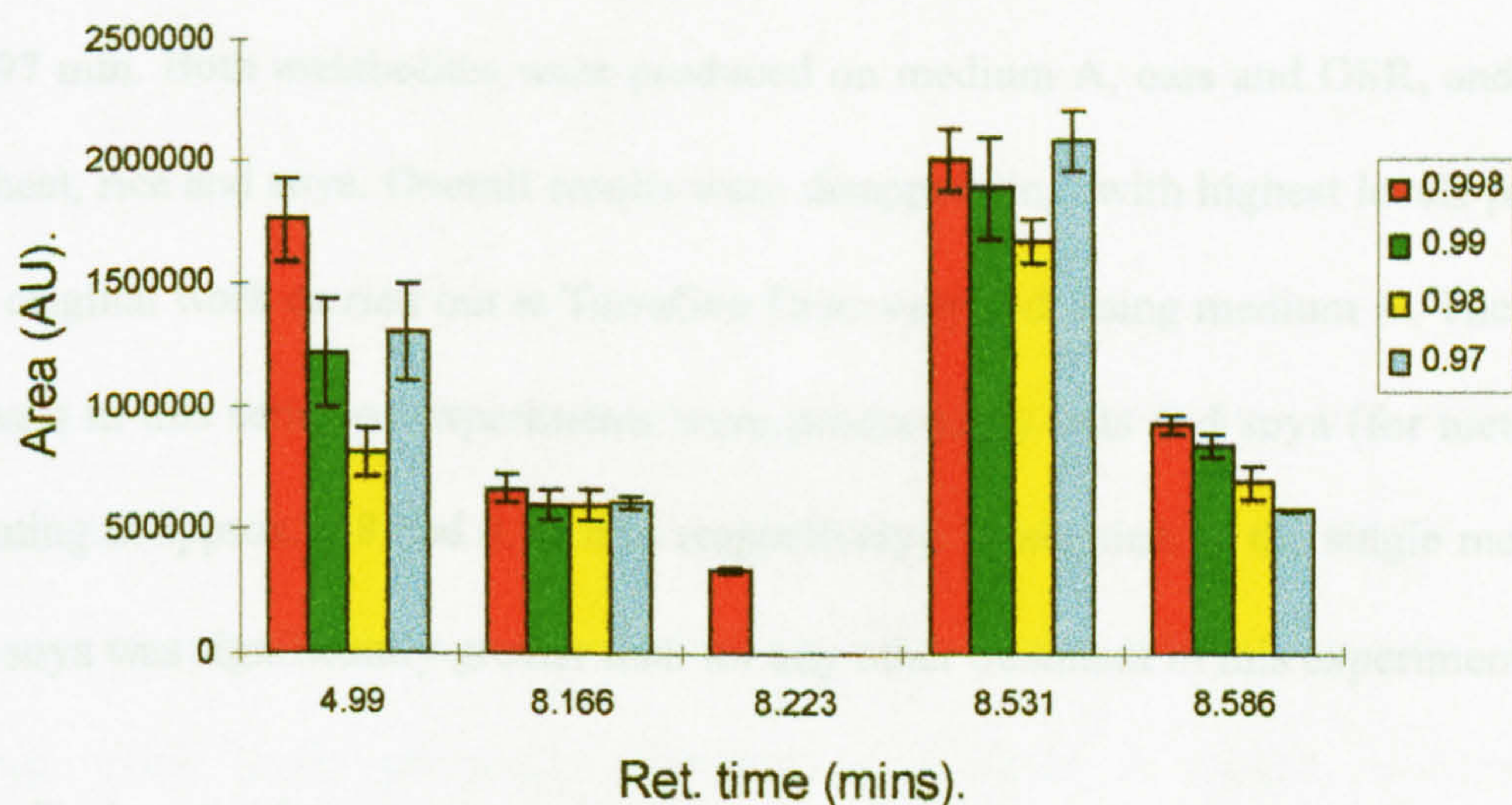


Figure 4.9. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *C. cassiicola* grown on (e) rice (f) soya. Bars are standard error of mean. Legend shows a_w range.

Many of the results were subject to high errors, so that an apparent variation was often not significant (as indicated by overlapping error bars). It is clear however that *C. cassiicola* was able to produce significant amounts of metabolites at the lowest a_w levels employed.

(c) Target metabolites.

Target metabolites for *C. cassiicola* were represented by a family of three peaks eluting at 4.72, 4.82 and 4.92 min, all with very similar spectrum index plots (see Appendix 4). Where present, target metabolites are shown in each metabolite profile and also in the comparative chart (Fig. 4.10.). Of the three metabolites selected, two appear in some profiles. These are metabolites eluting at 4.79 – 4.80 min and 4.945 – 4.97 min. Both metabolites were produced on medium A, oats and OSR, and one on wheat, rice and soya. Overall results were disappointing, with highest levels produced in original work carried out at TerraGen Discovery Ltd. using medium A. The highest levels in this series of experiments were produced by oats and soya (for metabolites eluting at approx. 4.8 and 4.95 min respectively). Production of the single metabolite in soya was significantly greater than for any other treatment in this experiment.

2. *D. dematioidea*

(a) Effects of medium type on secondary metabolite profiles.

Secondary metabolite profiles for *D. dematioidea* are shown in Figs 4.11. (a) – (d). These profiles show the complete range of peaks obtained for this organism. As with *C. cassiicola*, the profiles are strikingly different in both range and levels of metabolites. Medium A and wheat followed reasonably similar patterns, with perhaps

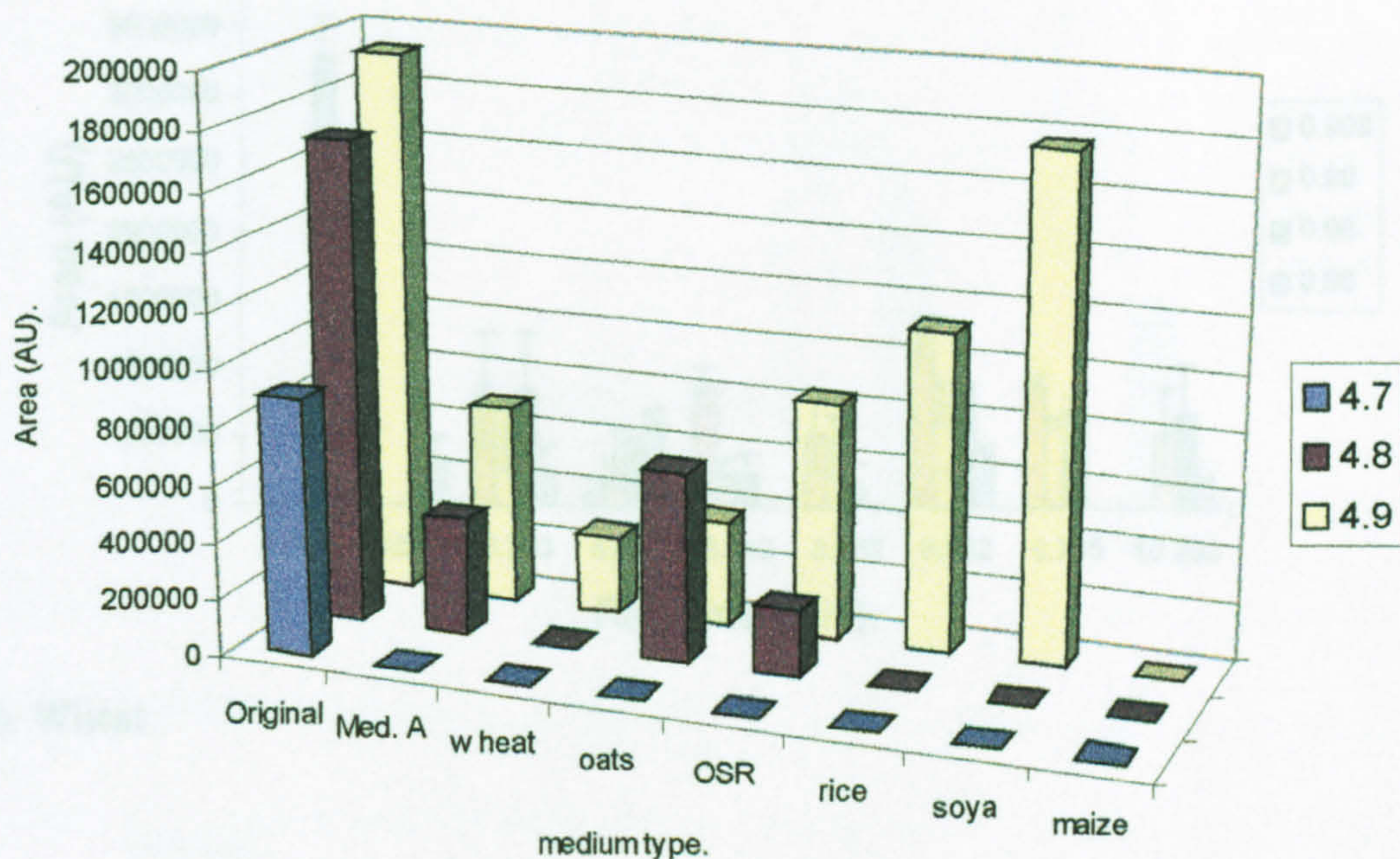
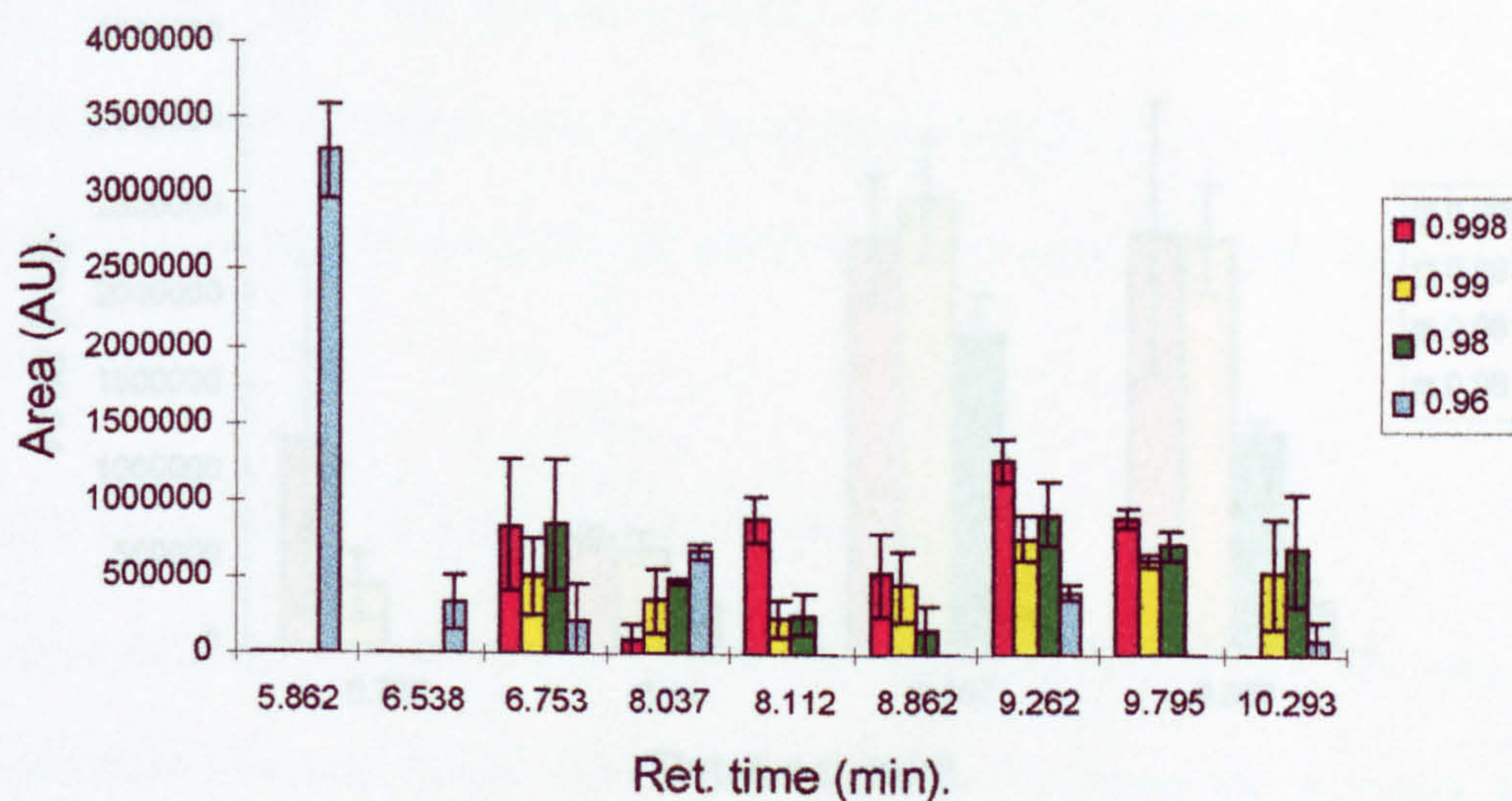


Figure 4.10. Comparison of target metabolite levels (mean peak area measured in absorption units) obtained for *C. cassiicola* grown on solid substrates. Amounts shown represent the maximum level obtained for each substrate. "Original" refers to target peak levels previously obtained for medium A (single measurements). Metabolites are identified in legend by elution time (min.). OSR = oil seed rape.

a. Medium A.



b. Wheat.

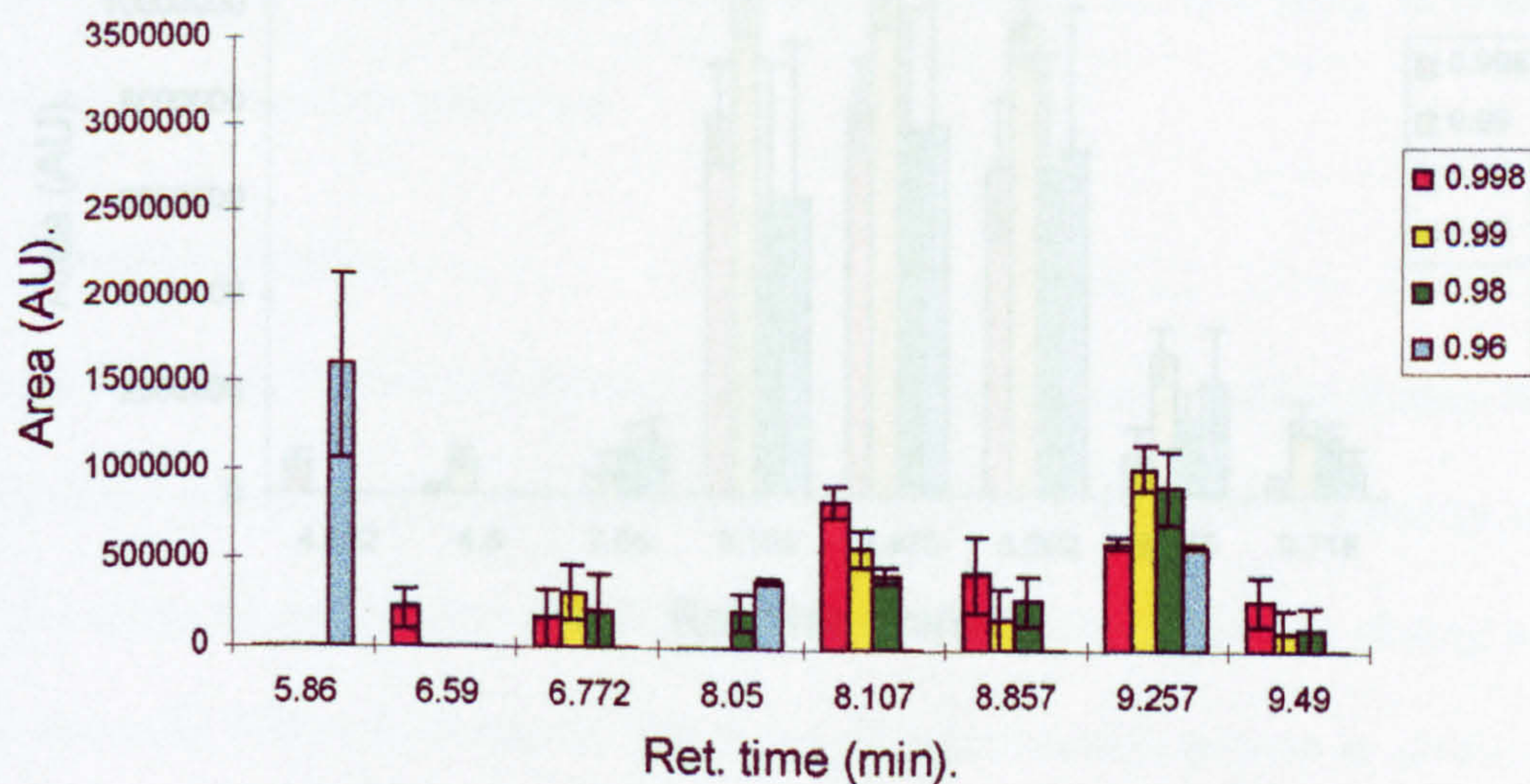
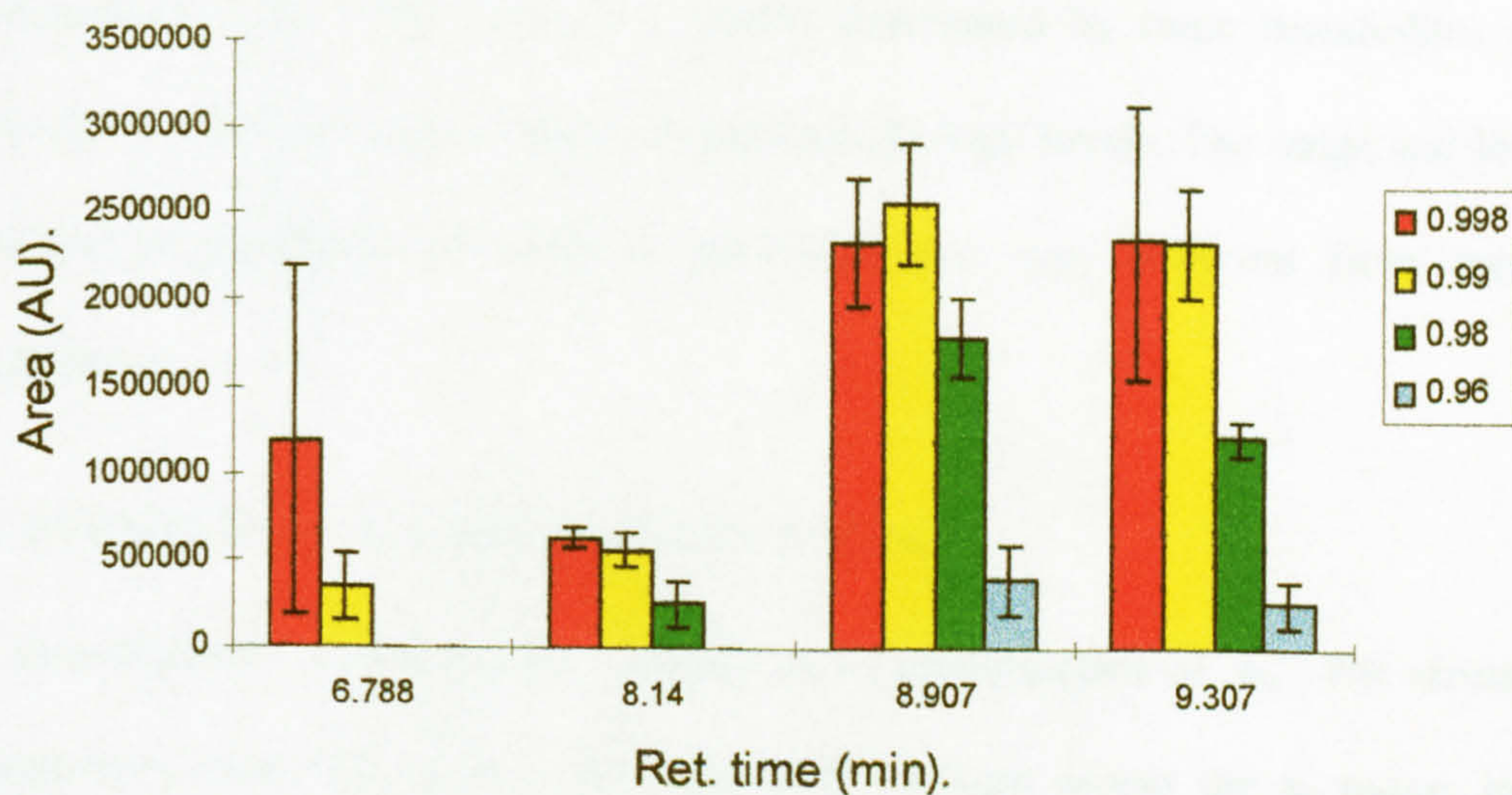


Figure 4.11. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *D. dematioidea*. Bars are standard error of mean. Legend shows a_w range.

c. Oats.



d. Oil seed rape.

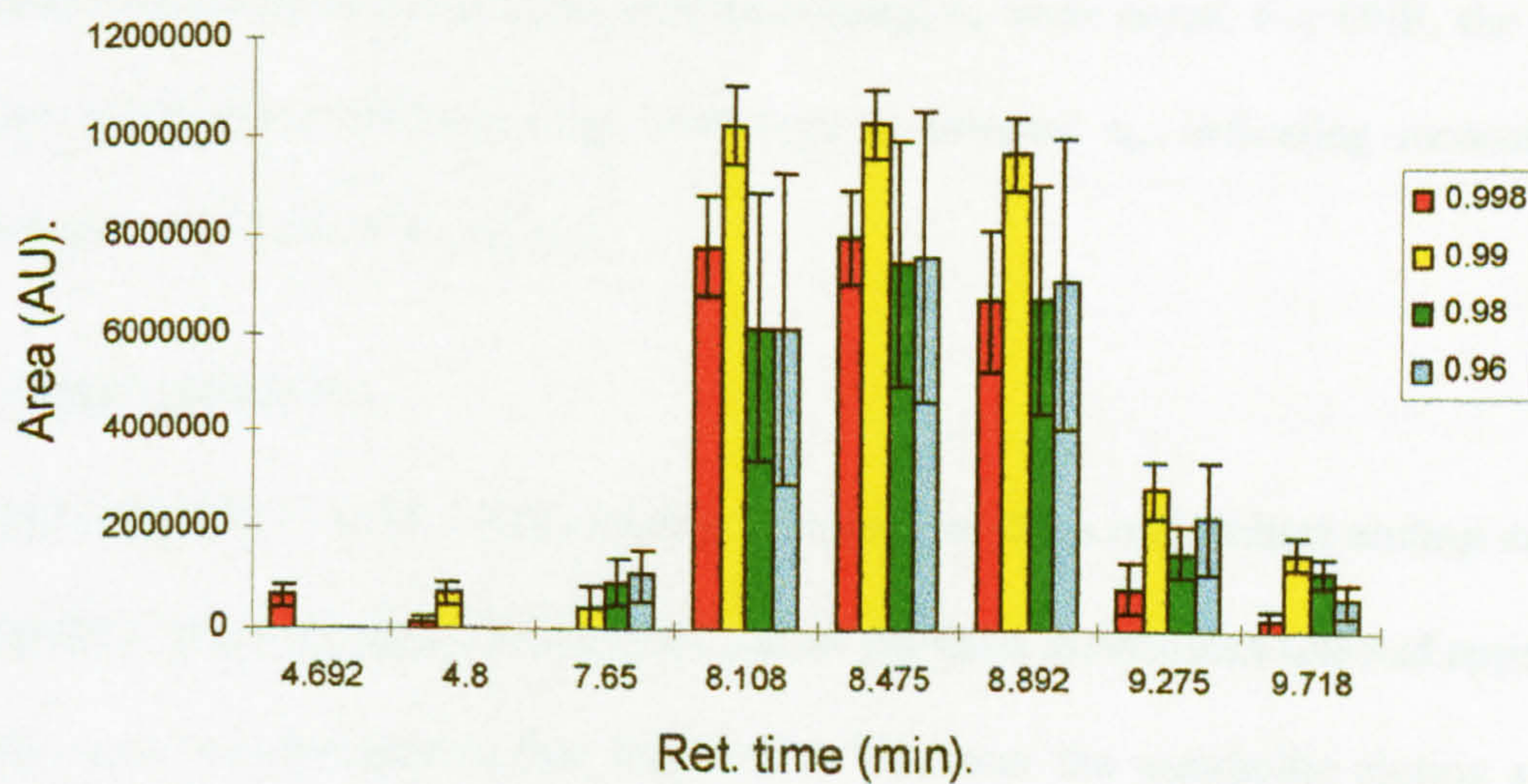


Figure 4.11. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *D. dematioidea*. Bars are standard error of mean. Legend shows a_w range.

slightly lower levels for the latter. Both showed a wider range of metabolites compared to oats. OSR showed a profile dominated by three metabolites eluting between 8.1 and 8.9 min produced at particularly high levels. The range and levels of metabolites produced on OSR in particular was very different from the other treatments.

(b) Effects of a_w on secondary metabolite profiles.

D. dematioidea was not strongly influenced by manipulation of a_w . For virtually all metabolites observed, levels were reasonably uniform across the a_w range, in most cases declining moderately with lowered a_w . The strongest response seemed to occur in oats where significant declines with decreasing a_w were noted. For OSR, the three major metabolites exhibited large error bars at lowered a_w , indicating inconsistent production by replicate treatments.

(c) Target metabolites.

Target peaks for *D. dematioidea* were represented as three metabolites eluting at 4.6, 6.4 and 6.7 min (see Appendix 4). Only one of the three metabolites selected appeared in this series of experiments (see Fig. 4.12.). This was the metabolite eluting at 6.7 min (actually 6.75 – 6.79 min in this series of experiments), present in profiles for medium A, wheat and oats. However, the metabolite was present at levels significantly lower than previously obtained for medium A.

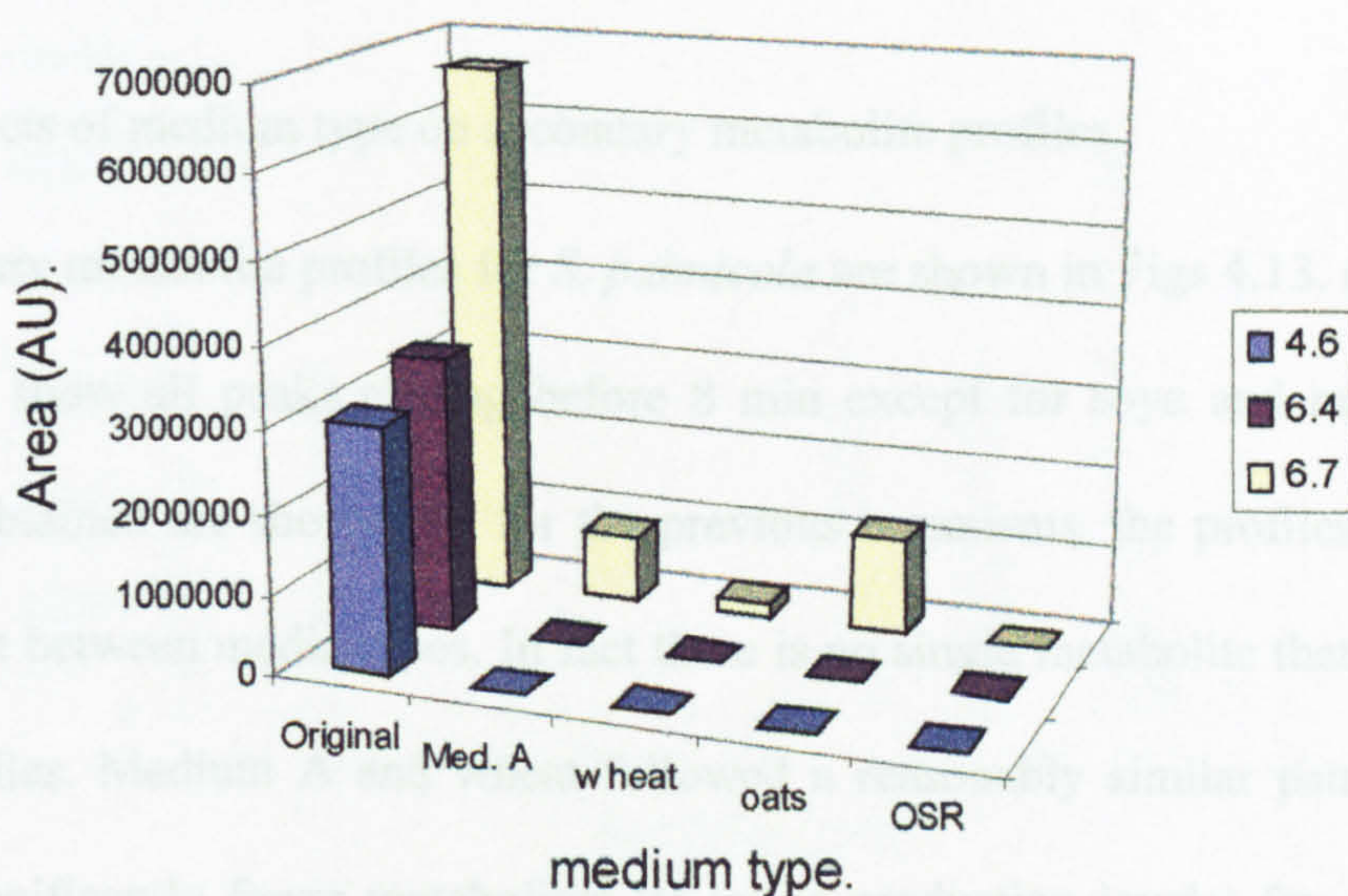


Figure 4.12. Comparison of target metabolite levels (mean peak area measured in absorption units) obtained for *D. dematioidea* grown on solid substrates. Amounts shown represent the maximum level obtained for each substrate. "Original" refers to target peak levels previously obtained for medium A (single measurements). Metabolites are identified in legend by elution time (min.). OSR = oil seed rape.

3. *S. palmicola*.

(a) Effects of medium type on secondary metabolite profiles.

Secondary metabolite profiles for *S. palmicola* are shown in Figs 4.13. (a) – (f). These profiles show all peaks eluting before 8 min except for soya and maize where all peaks obtained are shown. As for the previous organisms, the profiles are strikingly different between media types. In fact there is no single metabolite that is common to all profiles. Medium A and wheat followed a reasonably similar pattern and scale, with significantly fewer metabolites (at lower production levels) for oats and OSR. Profiles for soya and maize are entirely different from the others. *S. palmicola* did not produce any secondary metabolites when grown on rice.

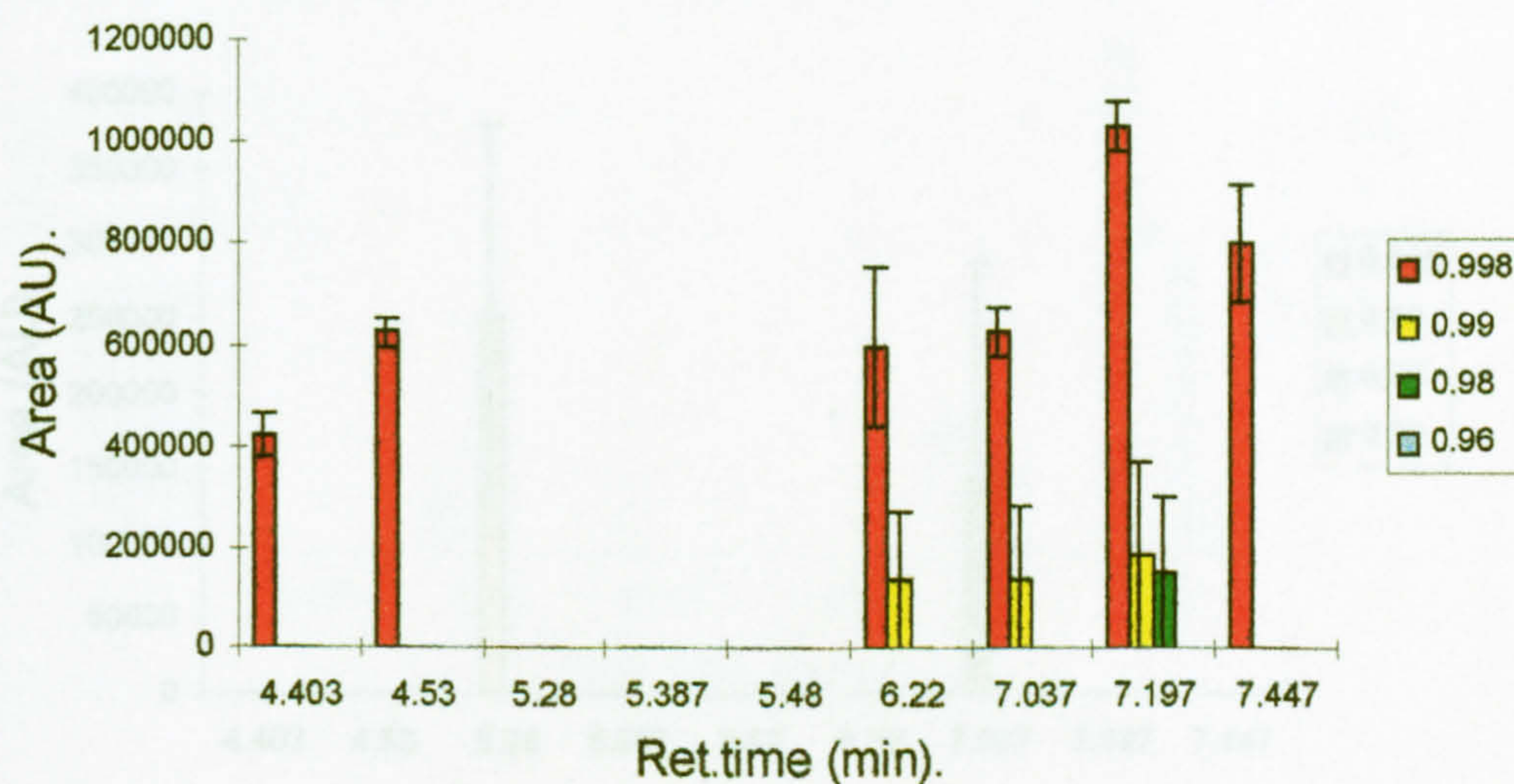
(b) Effects of a_w on secondary metabolite profiles.

Of all the organisms tested, *S. palmicola* responded most negatively to manipulation of a_w . As a_w was lowered there was a striking decrease in secondary metabolite production. Only two metabolites (7.037 min in wheat and 5.48 min in OSR) showed levels at 0.99 a_w that were not significantly lower than at 0.998 a_w . Only four metabolites were represented at 0.98 a_w and only one (8.57 min in maize) was represented at 0.97 a_w .

(c) Target metabolites.

Target metabolites for *S. palmicola* were represented as metabolites eluting at 4.4, 5.4, 6.0, 6.2 and 7.0 min. Of these six metabolites, two appeared in this series of

a. Medium A.



b. Wheat.

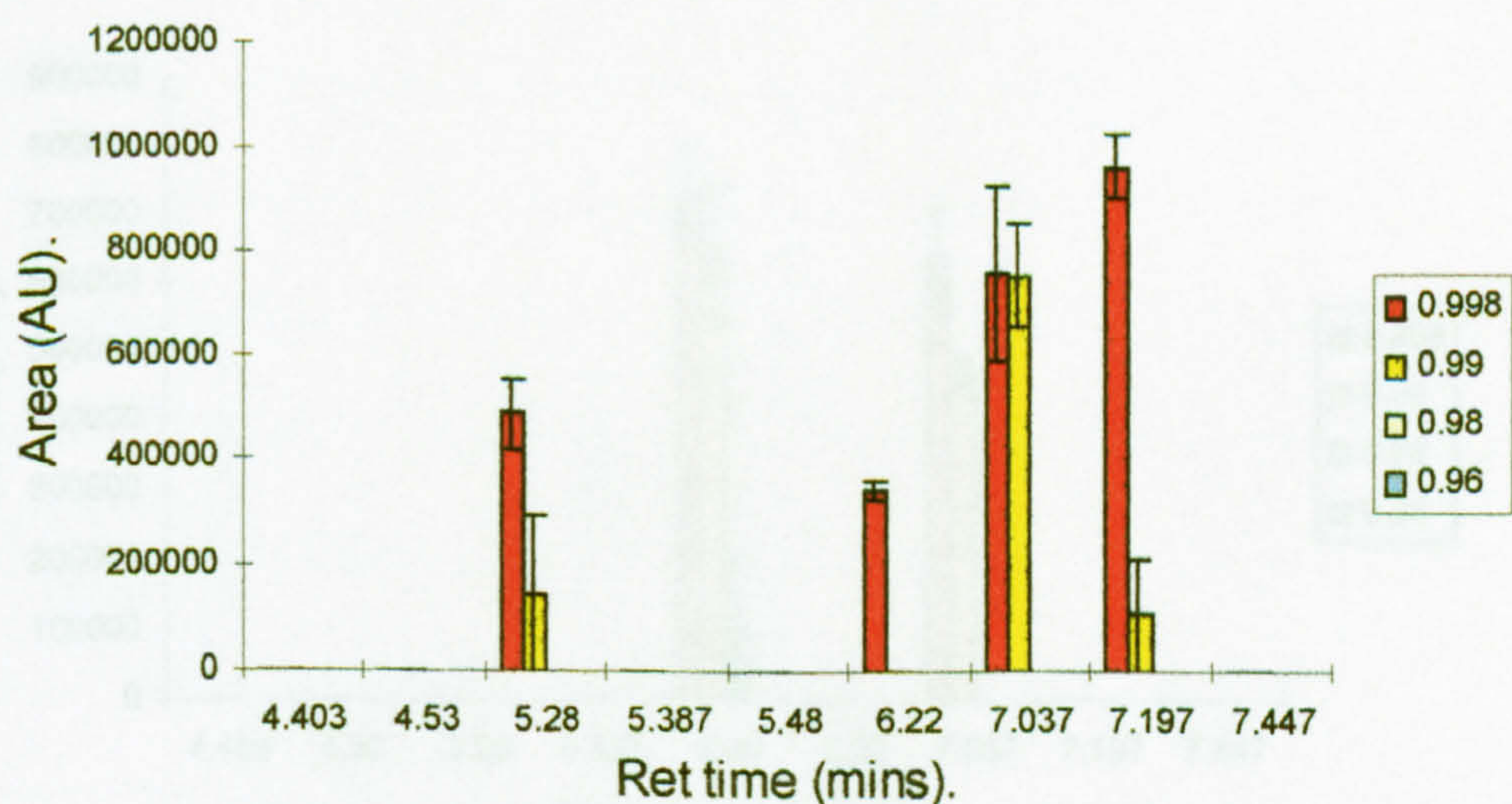
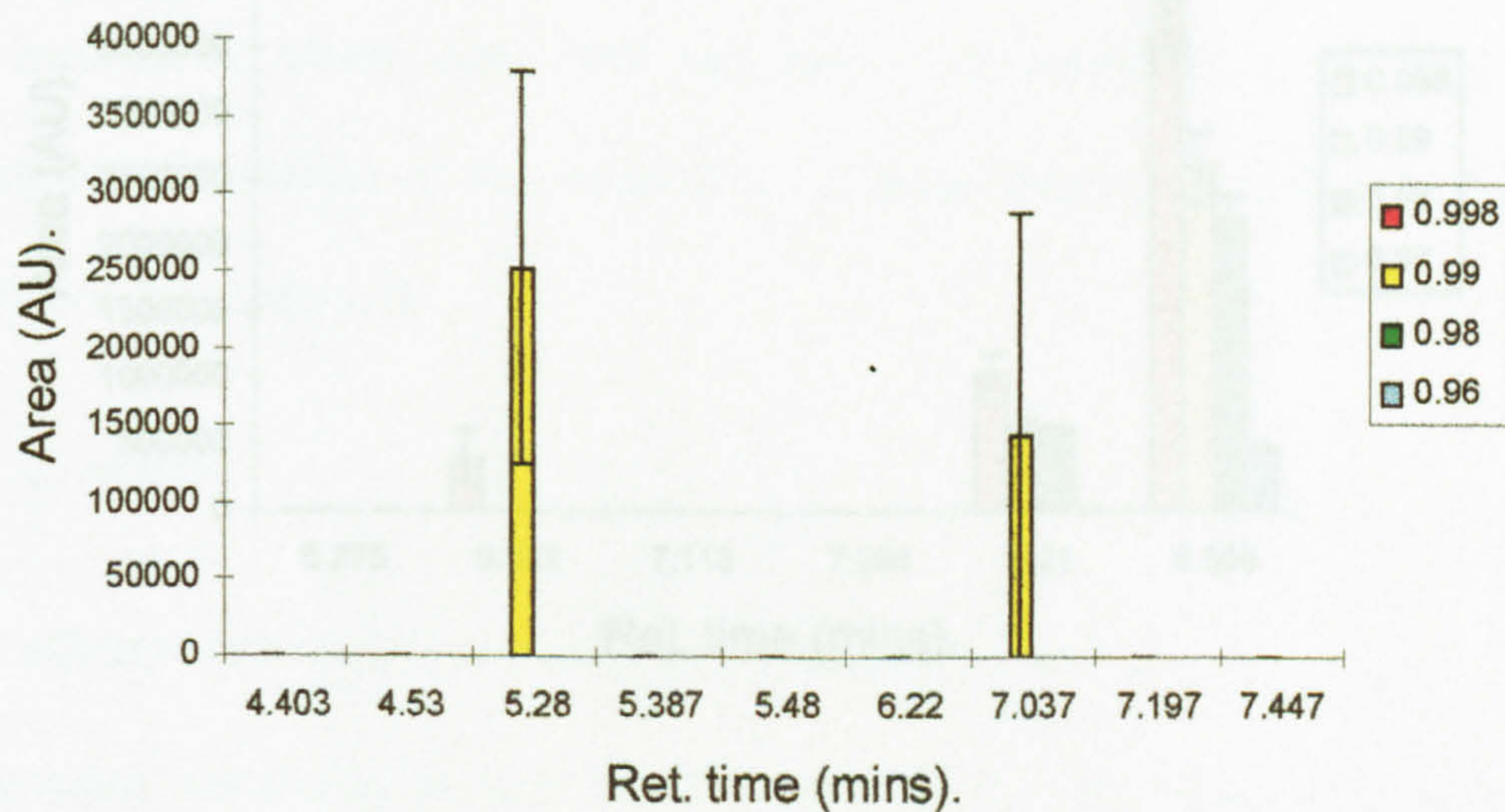


Figure 4.13. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *S. palmicola*. Bars are standard error of mean. Legend shows a_w range.

c. Oats.



d. Oil seed rape.

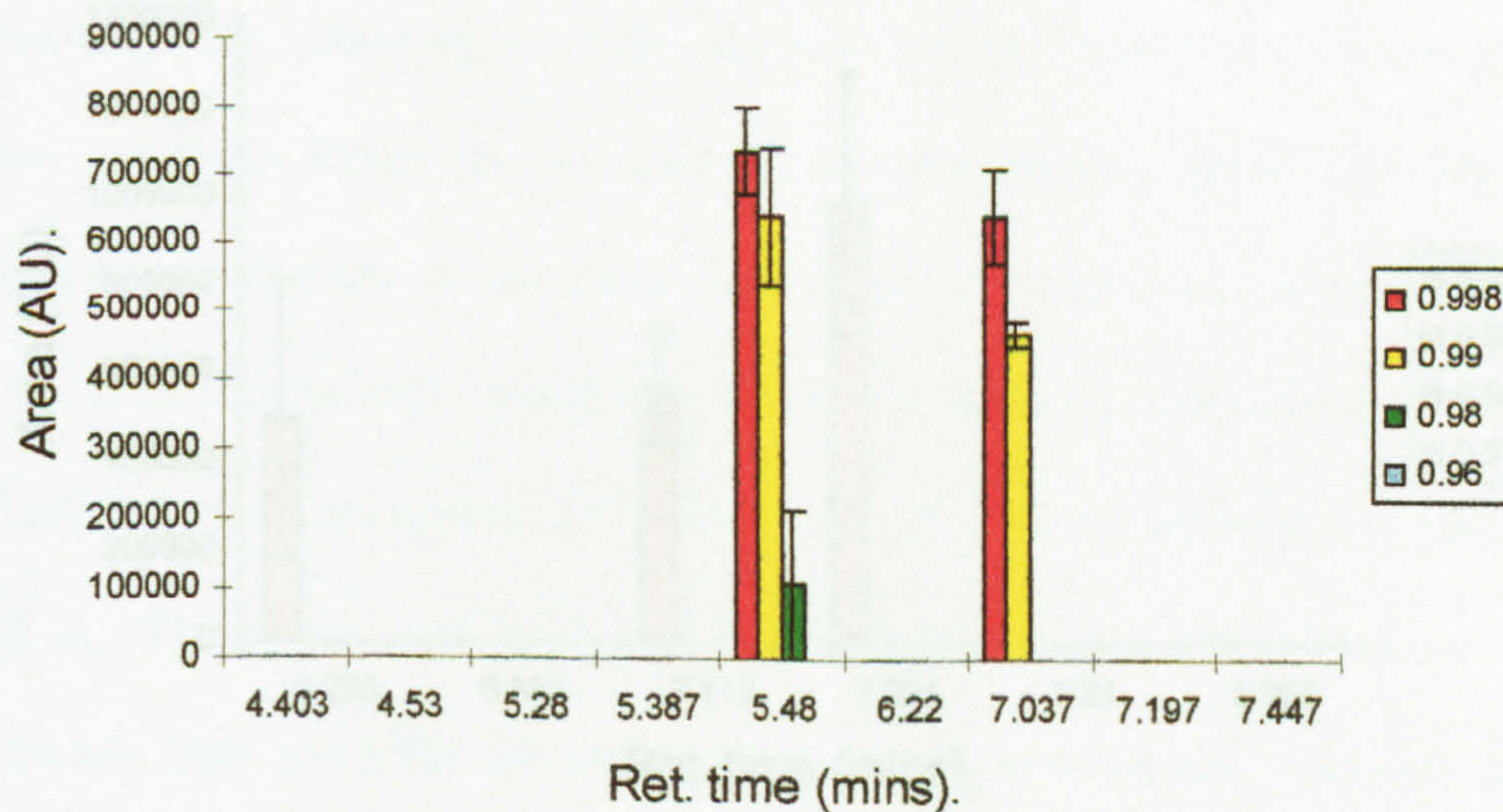


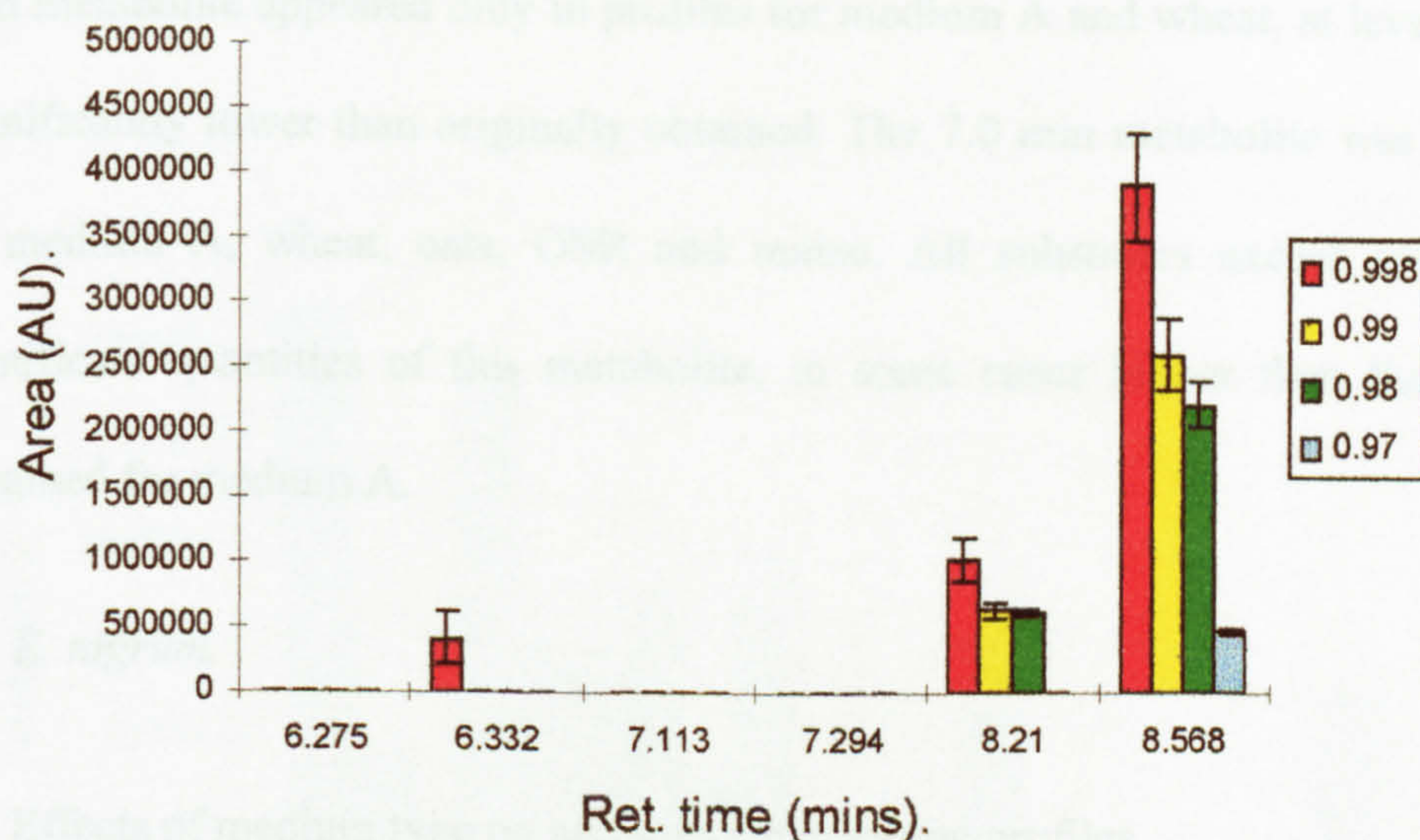
Figure 4.13. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs

Figure 4.13. (cont.). Secondary metabolite profiles over a range of a_w levels:

Graphs of HPLC peak area (absorption units) v. retention time (min.) for *S.*

palmicola. Bars are standard error of mean. Legend shows a_w range.

e. Soya.



f. Maize.

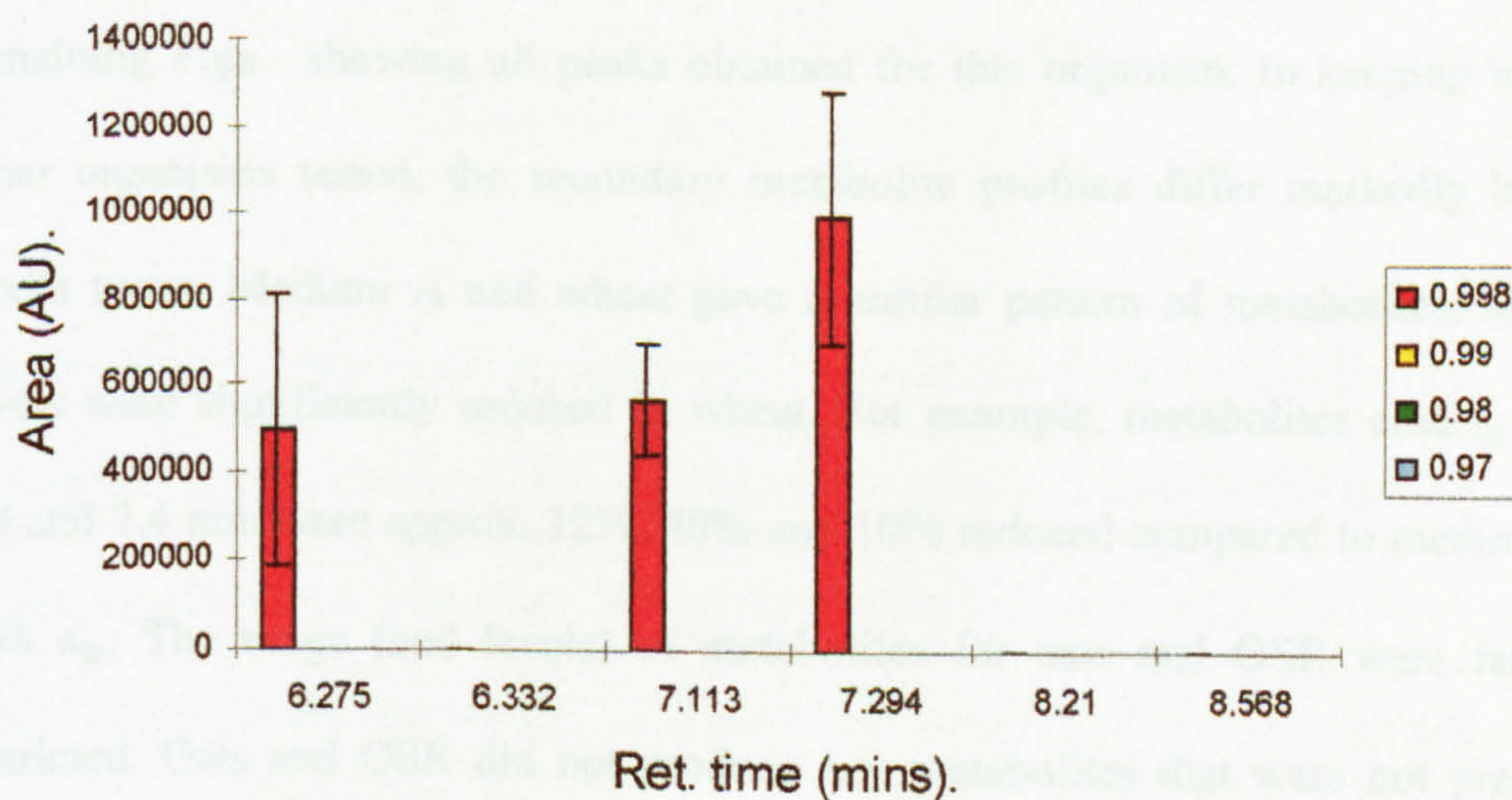


Figure 4.13. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *S. palmicola*. Bars are standard error of mean. Legend shows a_w range.

experiments (Fig. 4.14.). These are metabolites eluting at 6.2 and 7.0 min. The 6.2 min metabolite appeared only in profiles for medium A and wheat, at levels that were significantly lower than originally obtained. The 7.0 min metabolite was represented in medium A, wheat, oats, OSR and maize. All substrates except oats produced significant quantities of this metabolite, in some cases higher than that originally obtained for medium A.

4. *E. nigrum*.

(a) Effects of medium type on secondary metabolite profiles.

Secondary metabolite profiles for *E. nigrum* in the first series of experiments are shown in Figs. 4.15. (a) – (d). This shows all peaks eluting before 9 min, with the remaining Figs. showing all peaks obtained for this organism. In keeping with the other organisms tested, the secondary metabolite profiles differ markedly between media types. Medium A and wheat gave a similar pattern of metabolites, although levels were significantly reduced in wheat. For example, metabolites eluting at 6.4, 6.8 and 7.4 min were approx. 12%, 40% and 10% reduced compared to medium A at 0.98 a_w . The range (and levels) of metabolites for oats and OSR were far more restricted. Oats and OSR did not produce any metabolites that were not present in medium A and wheat.

Fig. 4.16 shows results for the series of experiments with various wheat flake treatments (it has been possible to keep the absorption scale the same for these profiles to allow direct comparison). The range and levels of metabolites produced

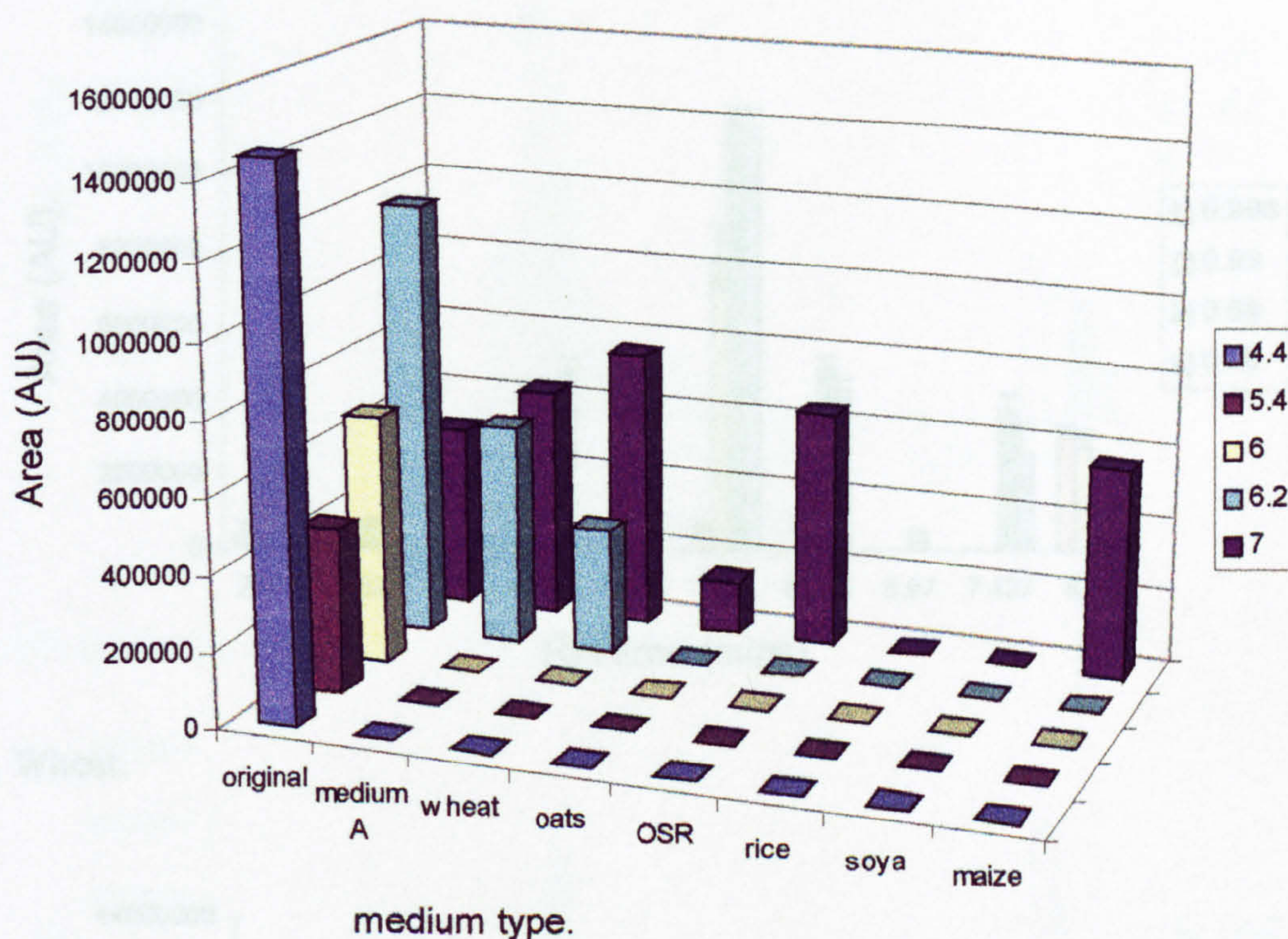
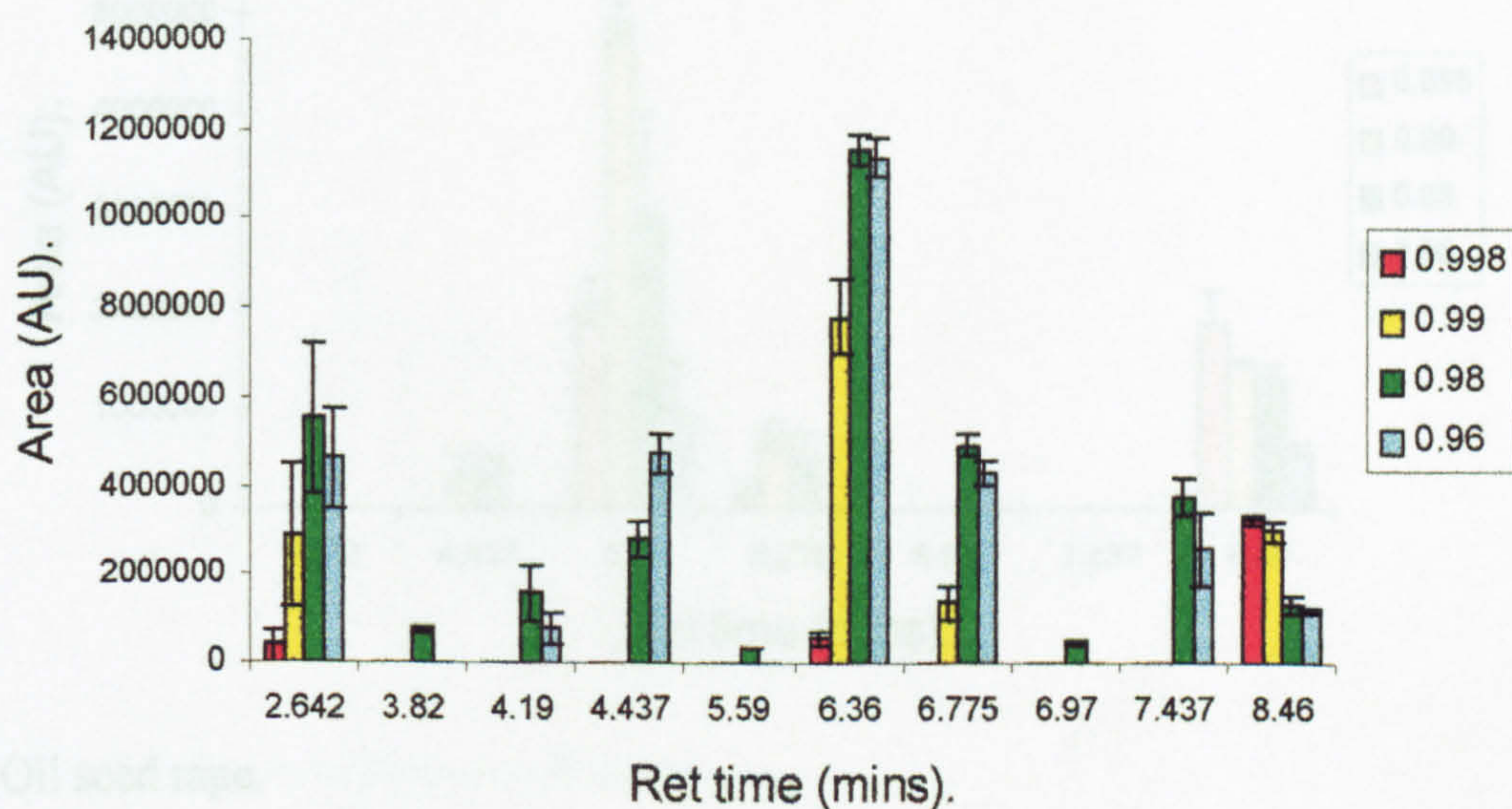


Figure 4.14. Comparison of target metabolite levels (mean peak area measured in absorption units) obtained for *S. palmicola* grown on solid substrates. Amounts shown represent the maximum level obtained for each substrate. "Original" refers to target peak levels previously obtained for medium A (single measurements). Metabolites are identified in legend by elution time (min.). OSR = oil seed rape.

a. Medium A.



b. Wheat.

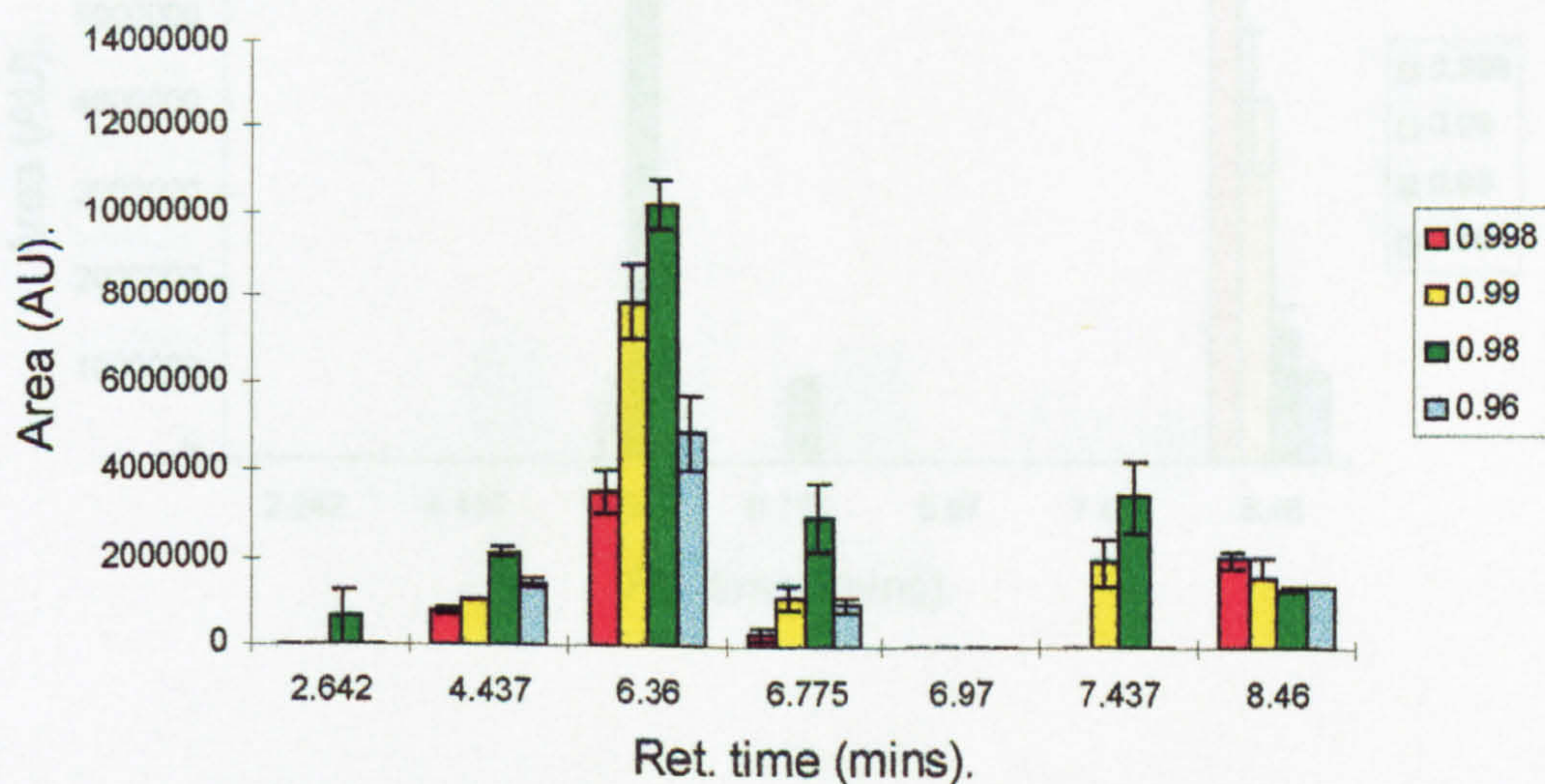
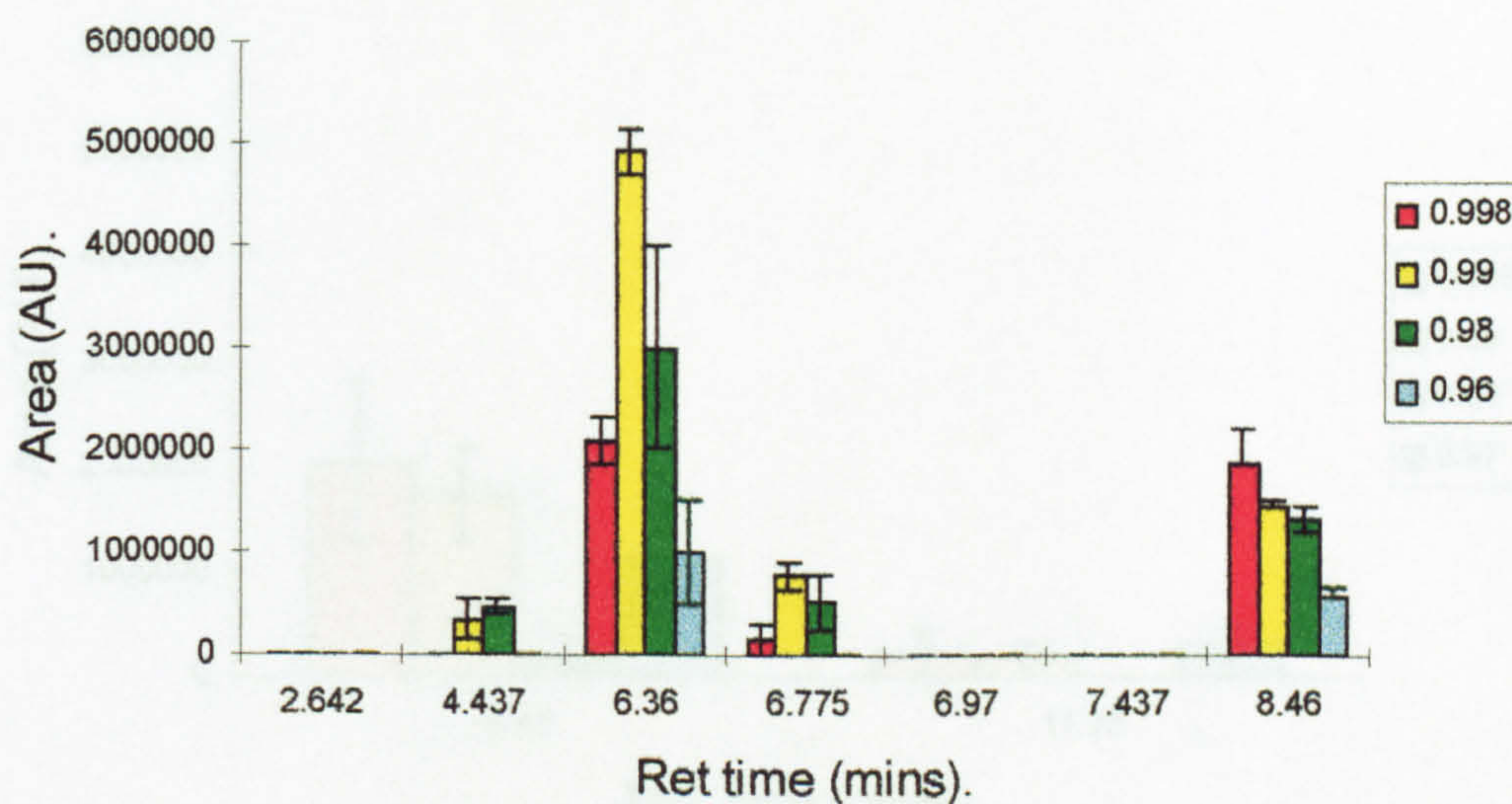


Figure 4.15. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *E. nigrum*. In graph a., metabolites eluting at 3.82, 4.19, 5.59, 6.77 and 6.97 min. have not been observed previously.

c. Oats.



d. Oil seed rape.

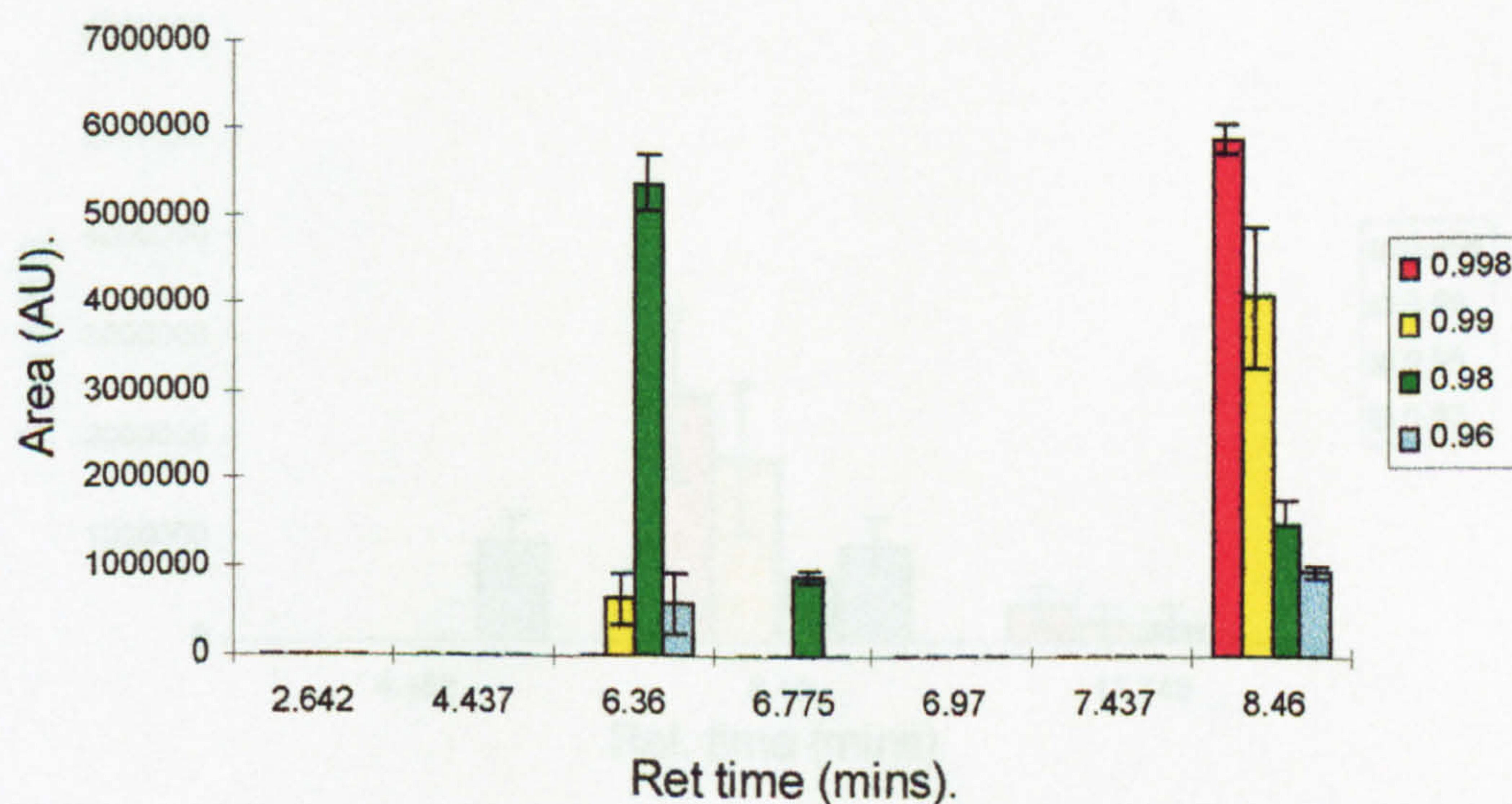
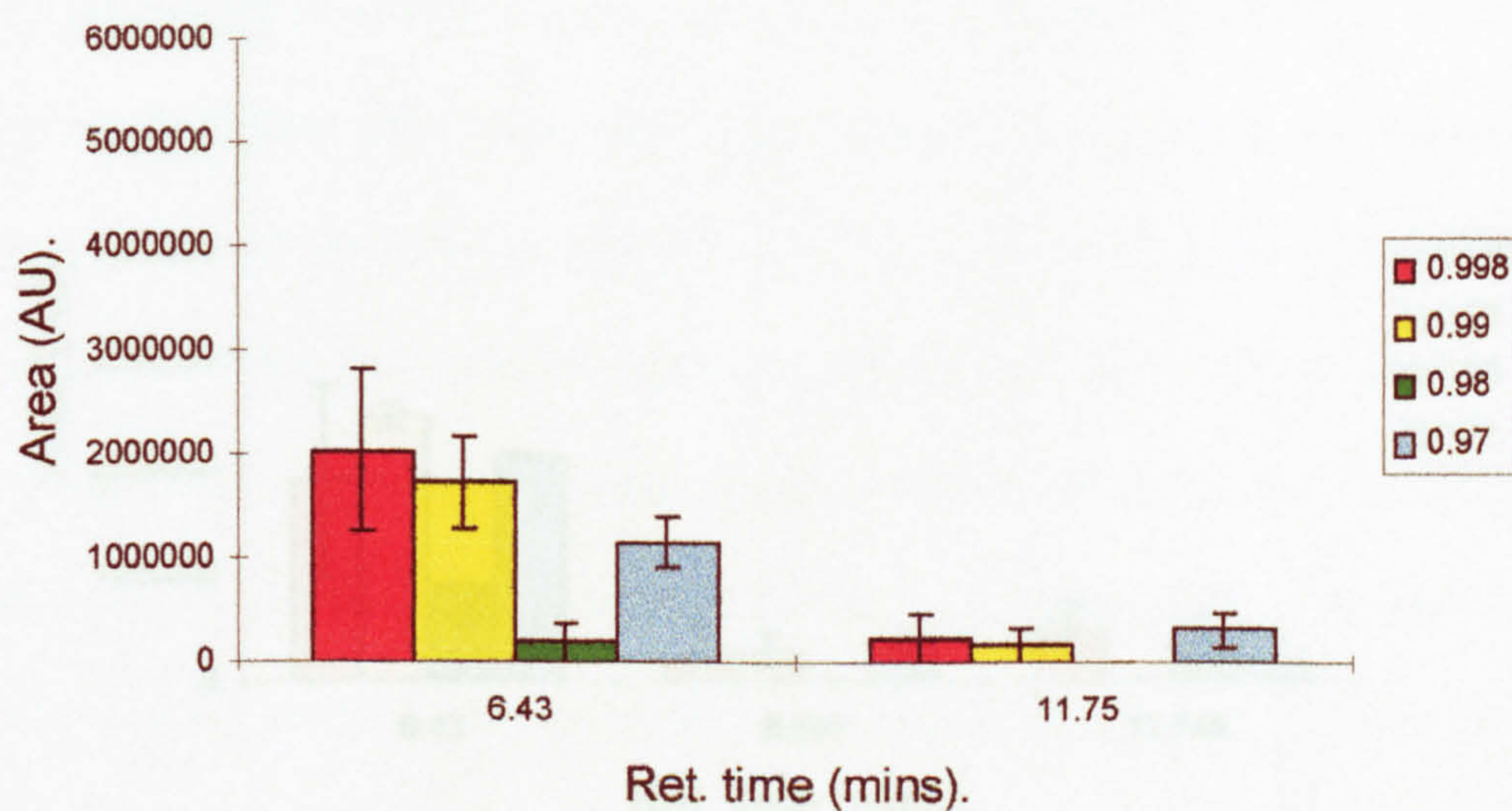


Figure 4.15. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *E. nigrum*. Bars are standard error of mean. Legend shows a_w range.

a. Wheat flakes.



b. Wheat flakes + nutrient supplement.

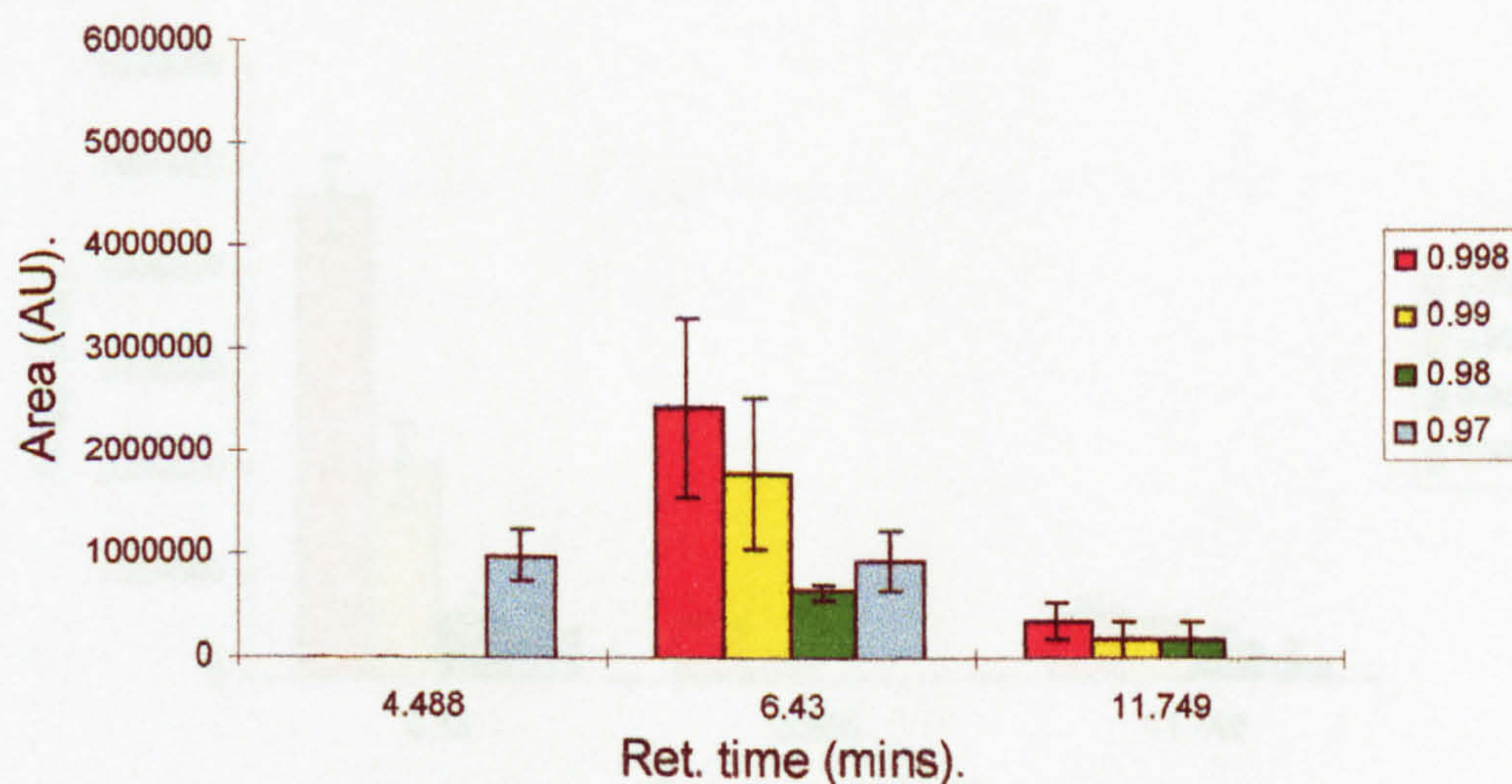
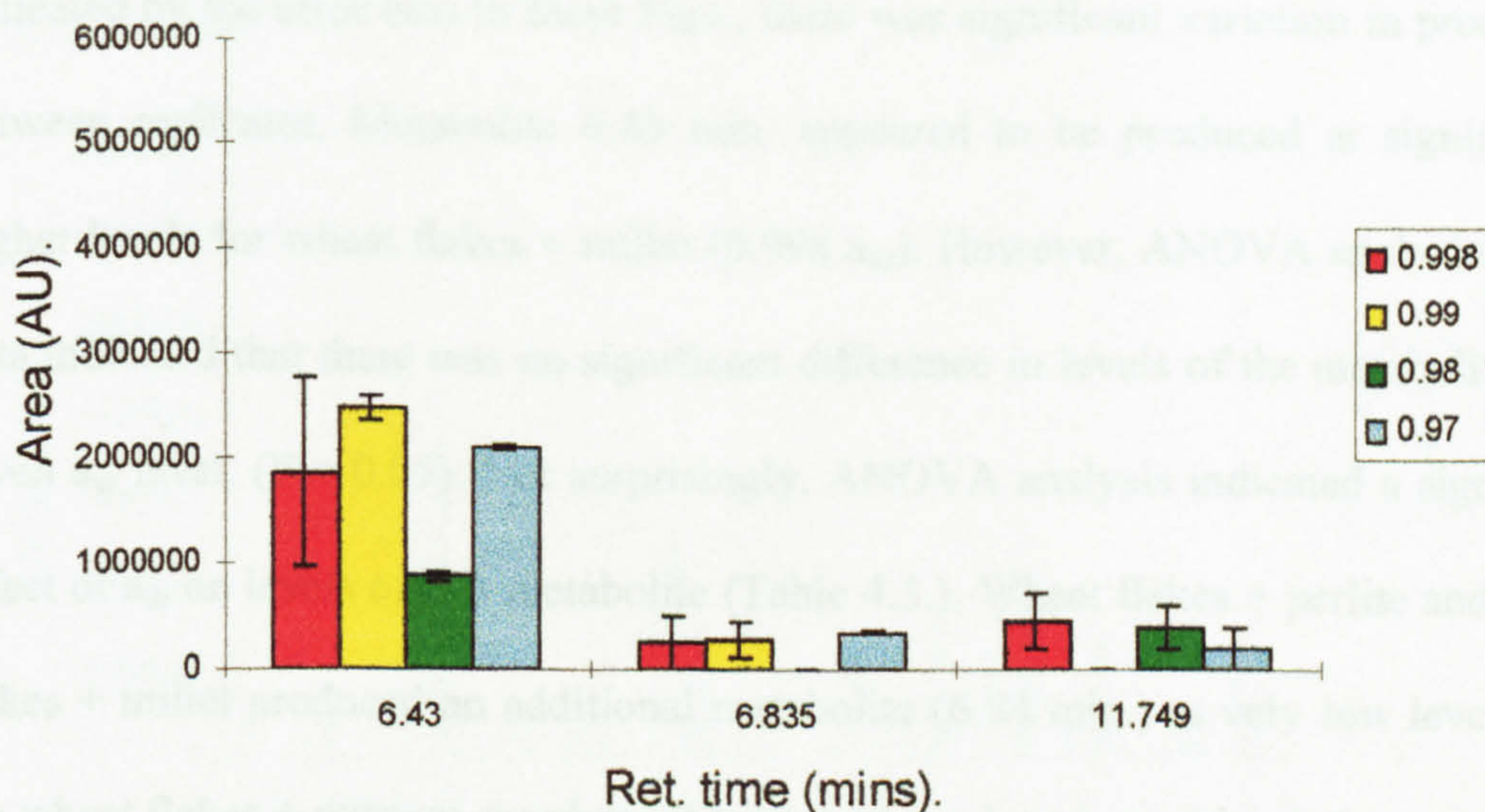


Figure 4.16. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *E. nigrum*. Bars are standard error of mean. Legend shows a_w range.

c. Wheat flakes + perlite



d. Wheat flakes + millet.

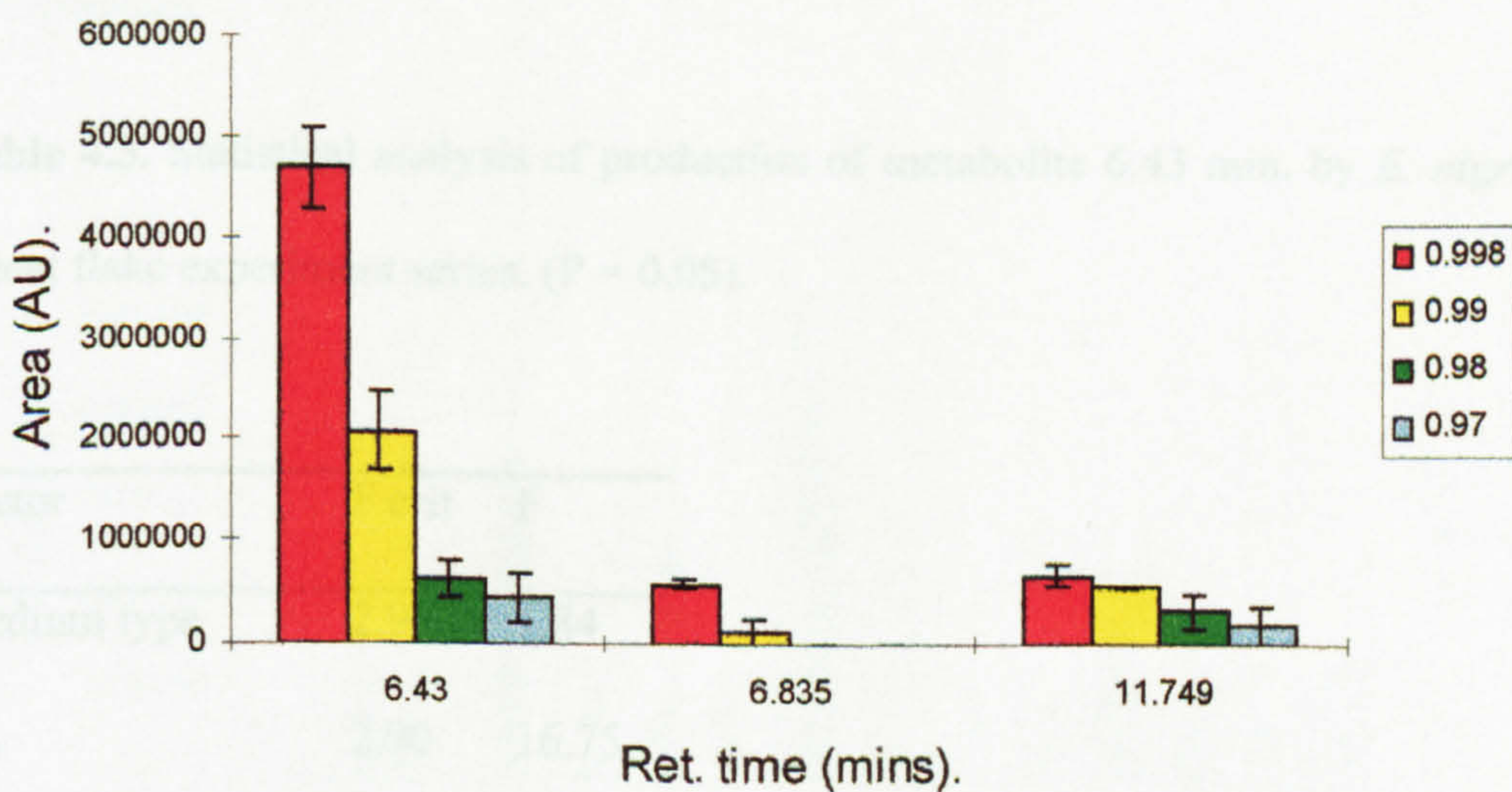


Figure 4.16. (cont.). Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *E. nigrum*. Bars are standard error of mean. Legend shows a_w range.

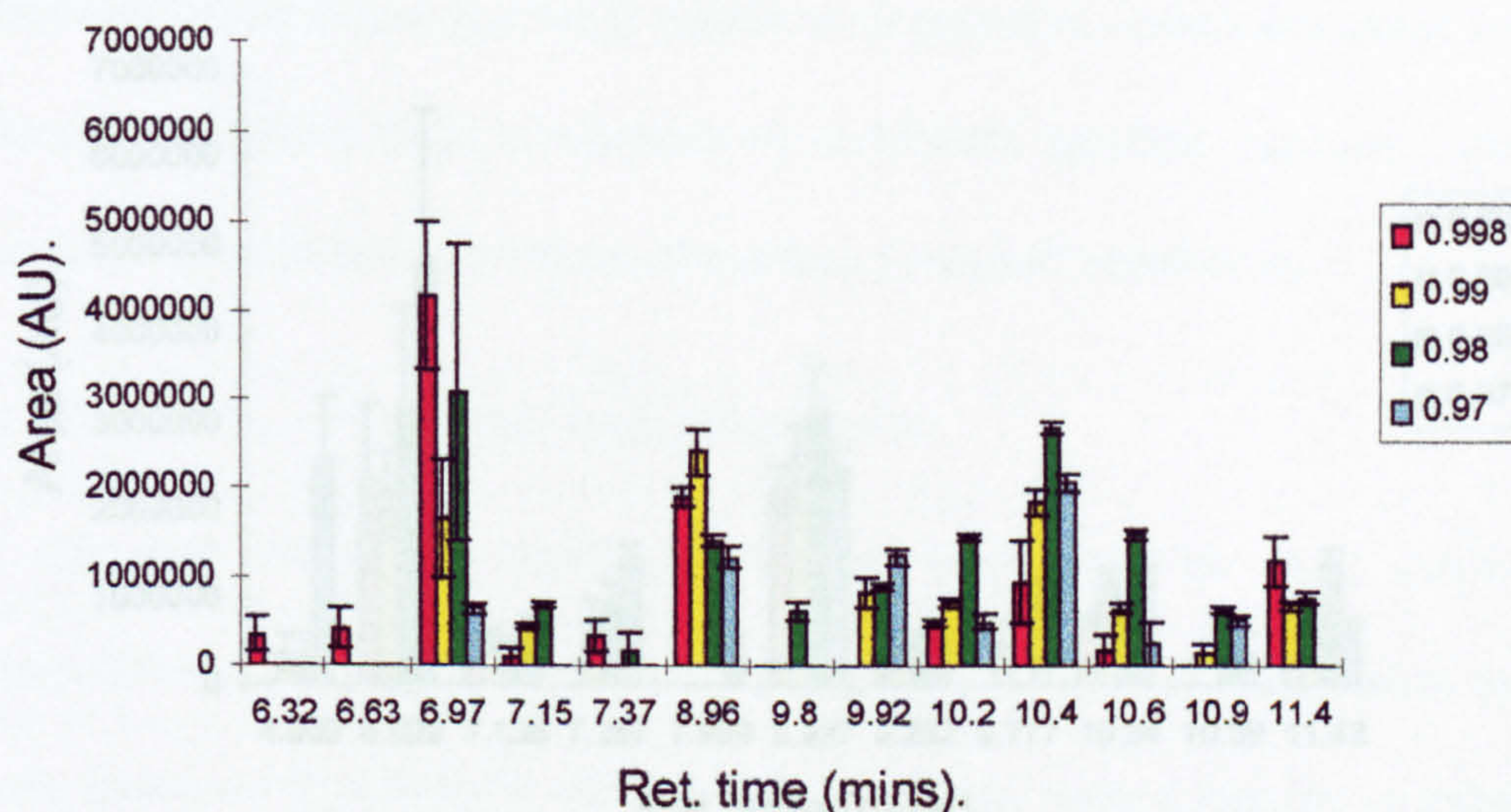
were much reduced compared to the previous series of experiments. Further, as indicated by the error bars in these Figs., there was significant variation in production between replicates. Metabolite 6.43 min. appeared to be produced at significantly higher levels for wheat flakes + millet (0.998 a_w). However, ANOVA analysis of this data indicated that there was no significant difference in levels of the metabolite for a given a_w level. ($P = 0.05$). Not surprisingly, ANOVA analysis indicated a significant effect of a_w on levels of this metabolite (Table 4.3.). Wheat flakes + perlite and wheat flakes + millet produced an additional metabolite (6.84 min.) at very low levels, and the wheat flakes + nutrient supplement treatment produced an early eluting metabolite (4.49 min.) only at 0.97 a_w .

Table 4.3. Statistical analysis of production of metabolite 6.43 min. by *E. nigrum* in wheat flake experiment series. ($P = 0.05$).

Factor	F crit	F
Medium type	2.90	1.84
A_w	2.90	16.75
A_w x medium type	3.07	2.19

Fig. 4.17. shows profiles for bulgar wheat, couscous, couscous + millet and shredded wheat. These profiles are very different from the preceding ones and are mainly characterised by a range of late eluting metabolites i.e. beyond 8.0 min. These

a. Bulgar wheat.



b. Couscous.

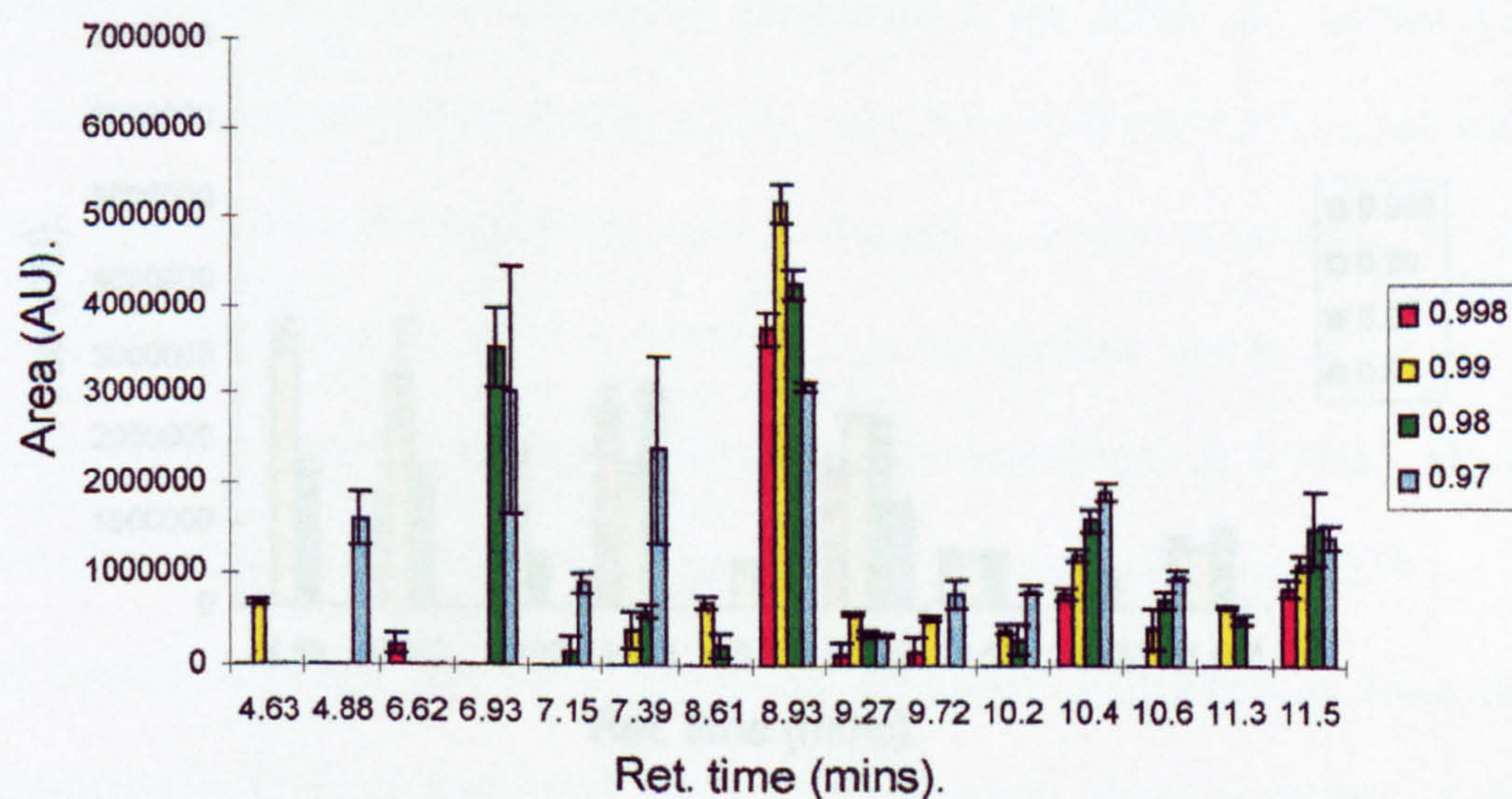
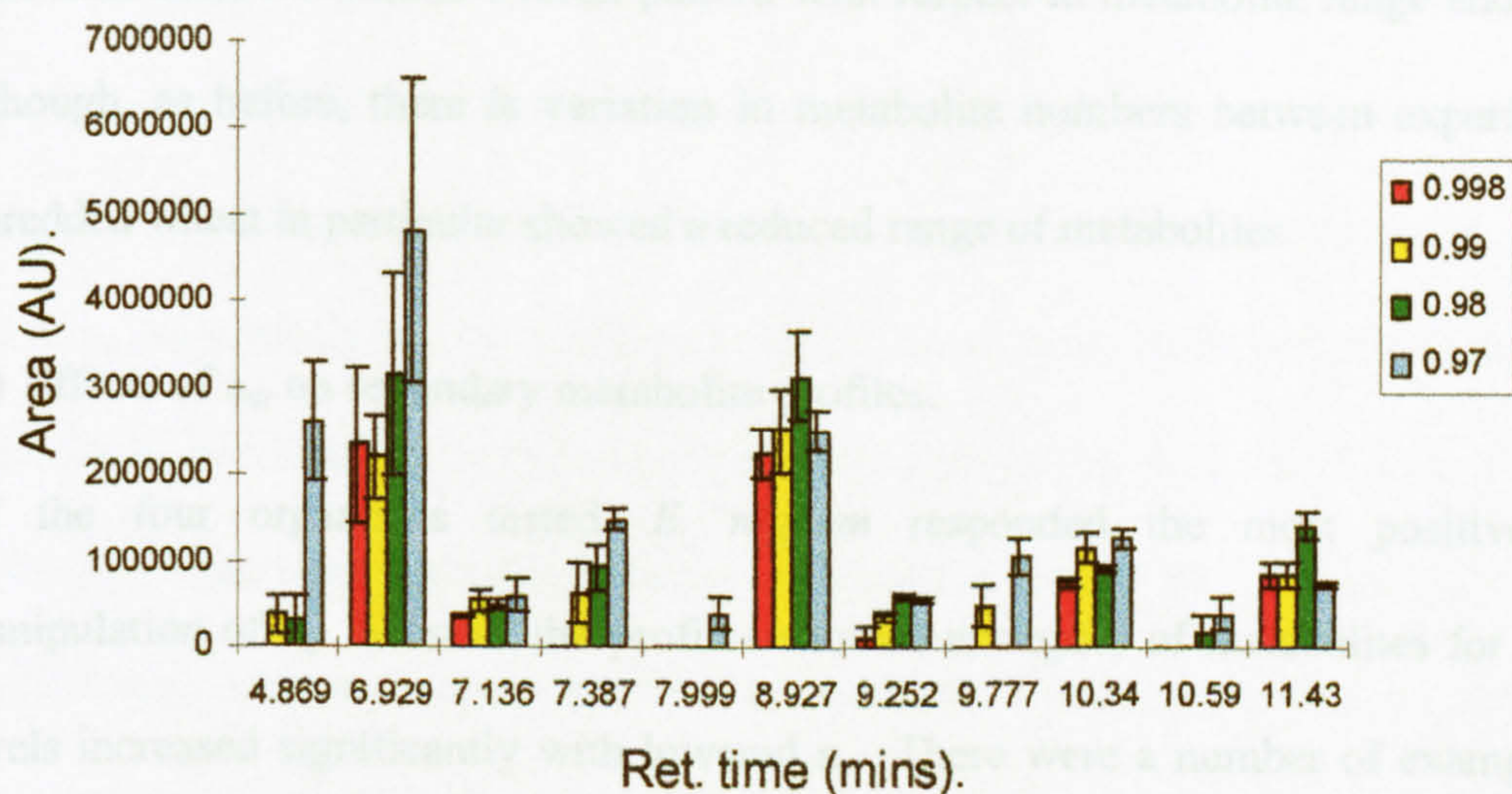


Figure 4.17. Secondary metabolite profiles over a range of a_w levels: Graphs of HPLC peak area (absorption units) v. retention time (min.) for *E. nigrum*. Bars are standard error of mean. Legend shows a_w range. Note: all elution times displaced +0.5 min relative to previous profiles.

c. Couscous + millet.



d. "Shredded wheat".

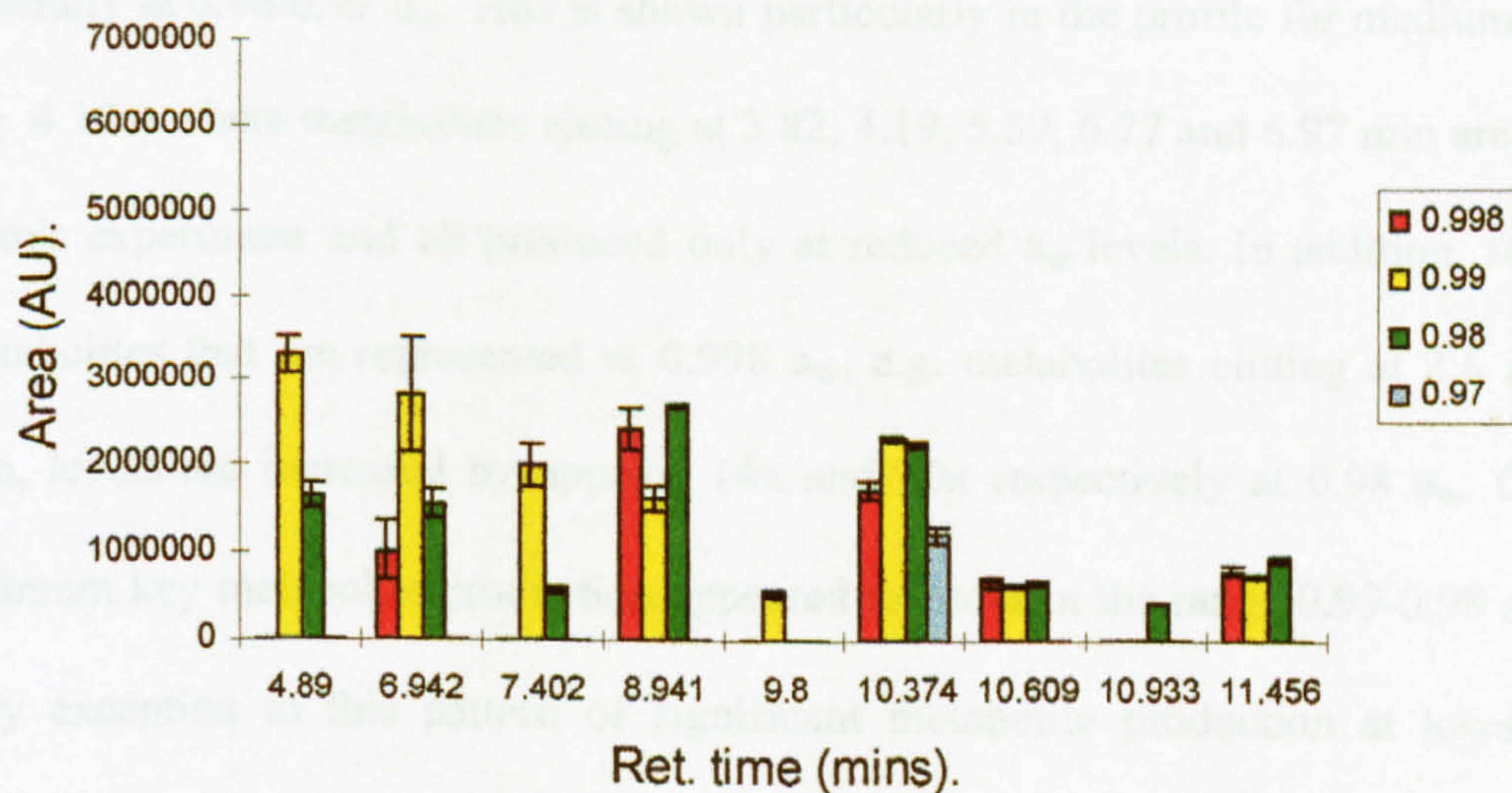


Figure 4.17. (cont.). Secondary metabolite profiles over a range of a_w levels:

Graphs of HPLC peak area (absorption units) v. retention time (min.) for *E. nigrum*.

Bars are standard error of mean. Legend shows a_w range. Note: all elution times displaced +0.5 min relative to previous profiles.

chemical species are less likely to be secondary metabolites, and may represent catabolic components of little pharmaceutical interest. Profiles for these four substrates follow a similar overall pattern with respect to metabolite range and levels although, as before, there is variation in metabolite numbers between experiments. Shredded wheat in particular showed a reduced range of metabolites.

(b) Effects of a_w on secondary metabolite profiles.

Of the four organisms tested, *E. nigrum* responded the most positively to manipulation of a_w . Most of the profiles showed examples of metabolites for which levels increased significantly with lowered a_w . There were a number of examples of metabolites that were only produced at lower a_w levels, and significant production generally at 0.96/0.97 a_w . This is shown particularly in the profile for medium A (see Fig. 4.15a) where metabolites eluting at 3.82, 4.19, 5.59, 6.77 and 6.97 min are unique to this experiment and all produced only at reduced a_w levels. In addition, for most metabolites that are represented at 0.998 a_w , e.g. metabolites eluting at 2.6 and 6.4 min, levels are increased by approx. 14x and 20x respectively at 0.98 a_w . Overall, optimum key metabolite production appeared to occur in the range 0.99-0.98 a_w . The only exception to this pattern of significant metabolite production at lowered a_w appeared to be in the wheat flakes series of experiments, which generally produced reduced levels of metabolites.

(c) Target metabolites.

Target metabolites for *E. nigrum* were selected as peaks eluting at 2.5, 5.6, 5.7, 6.4, 7.4 and 8.4 min. An additional shouldered peak, which consisted of two closely

eluting metabolites, was noted in these experiments and the first of the twin metabolites (eluting at 6.8 min.) was selected as a further peak of interest. Fig. 4.18. shows comparative production of target metabolites for all media types. Of the seven selected, three main metabolites appeared in this study. These were metabolites eluting at 6.4, 6.8, and 8.4 min. In the last four experiments these eluted at 6.9, 7.3 and 8.9 min. respectively due to a change in HPLC conditions. However, for clarity, they continue to be identified by the original elution times in Fig. 4.18. In addition to these, medium A and wheat produced target metabolites eluting at 2.5 and 7.4 min. In general, encouraging levels of metabolites were produced compared to the reference levels originally obtained by TerraGen Discovery Ltd. For example, the metabolite eluting at 6.4 min was increased by approx. 30% in medium A, and the metabolite eluting at 8.4 min was increased by approx. 17% in OSR.

Ultimately, *E. nigrum* and bulgar wheat were chosen as the model system (see Section 5.3.5.), and main target metabolites were those eluting at 6.9, 7.3 and 8.9 min, designated as metabolites 1, 2 and 3 respectively.

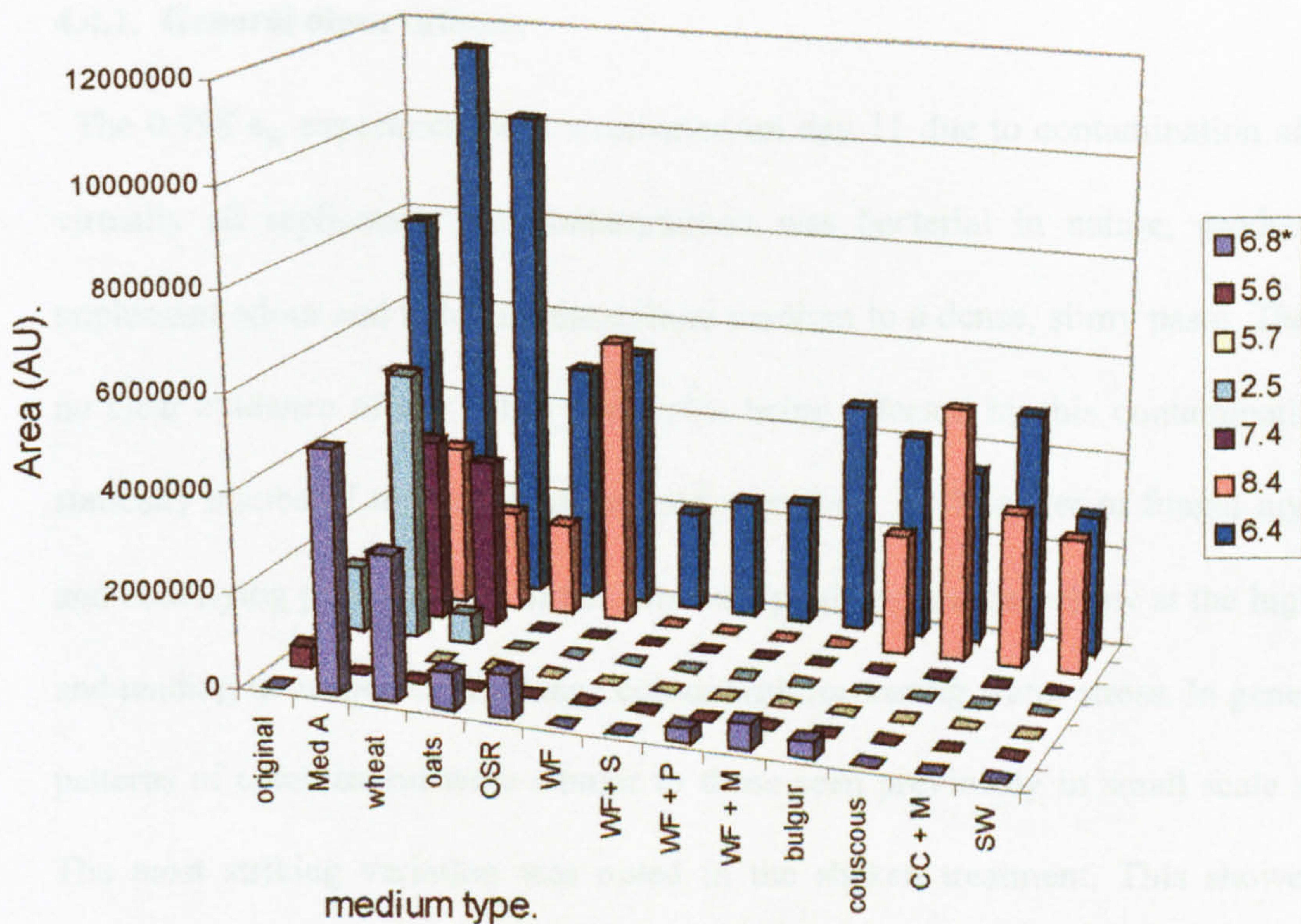


Figure 4.18. Comparison of target metabolite levels (mean peak area measured in absorption units) obtained for *E. nigrum* grown on solid substrates. Amounts shown represent the maximum level obtained for each substrate. "Original" refers to target peak levels previously obtained for medium A (single measurements). Metabolites are identified in legend by elution time (min.). 6.8* is an additional target peak obtained for some media. Key to treatments: WF = wheat flakes, S = supplement, P = perlite, M = millet, CC = couscous, SW = shredded wheat

4.4. TEMPORAL DEVELOPMENT OF METABOLITES BY *E. NIGRUM* GROWN ON BULGAR WHEAT.

4.4.1. General observations.

The 0.998 a_w experiment was terminated on day 11 due to contamination affecting virtually all replicates. The contamination was bacterial in nature, producing an unpleasant odour and reducing the culture medium to a dense, slimy paste. There was no clear evidence of any other treatments being affected by this contamination. All statically incubated treatments developed a uniform 100% cover of fungal mycelium and underlying pigmentation of the substrate particles; lemon yellow at the highest a_w and tending to deepen to an orange colour with increasing water stress. In general, the patterns of colonisation were similar to those seen previously in small scale studies. The most striking variation was noted in the shaken treatment. This showed very limited development of fungal mycelium, but had undergone a striking colour change to deep red by day 10 (see Plate 4.6).

4.4.2. Inoculation technique.

The use of pre-grown bulgar wheat as the inoculum in this study proved to be an efficient method, and in particular appeared to promote early and vigorous colonisation of the substrate at all a_w levels (see Plate 4.7). Further, the colour of pre-inoculated substrate grains facilitated direct assessment of the distribution of the inoculum in the substrate bulk (see Plate 4.8).



Plate 4.6. *E. nigrum* growing on bulgar wheat. Comparison of static (left) and shaken treatments. 0.998 a_w , day 3.



Plate 4.7. Colonisation by pre-grown bulgar wheat inoculum. 24 hrs. incubation, 25°C, 0.998 a_w . A – experimental treatment. B – x20 magnification.

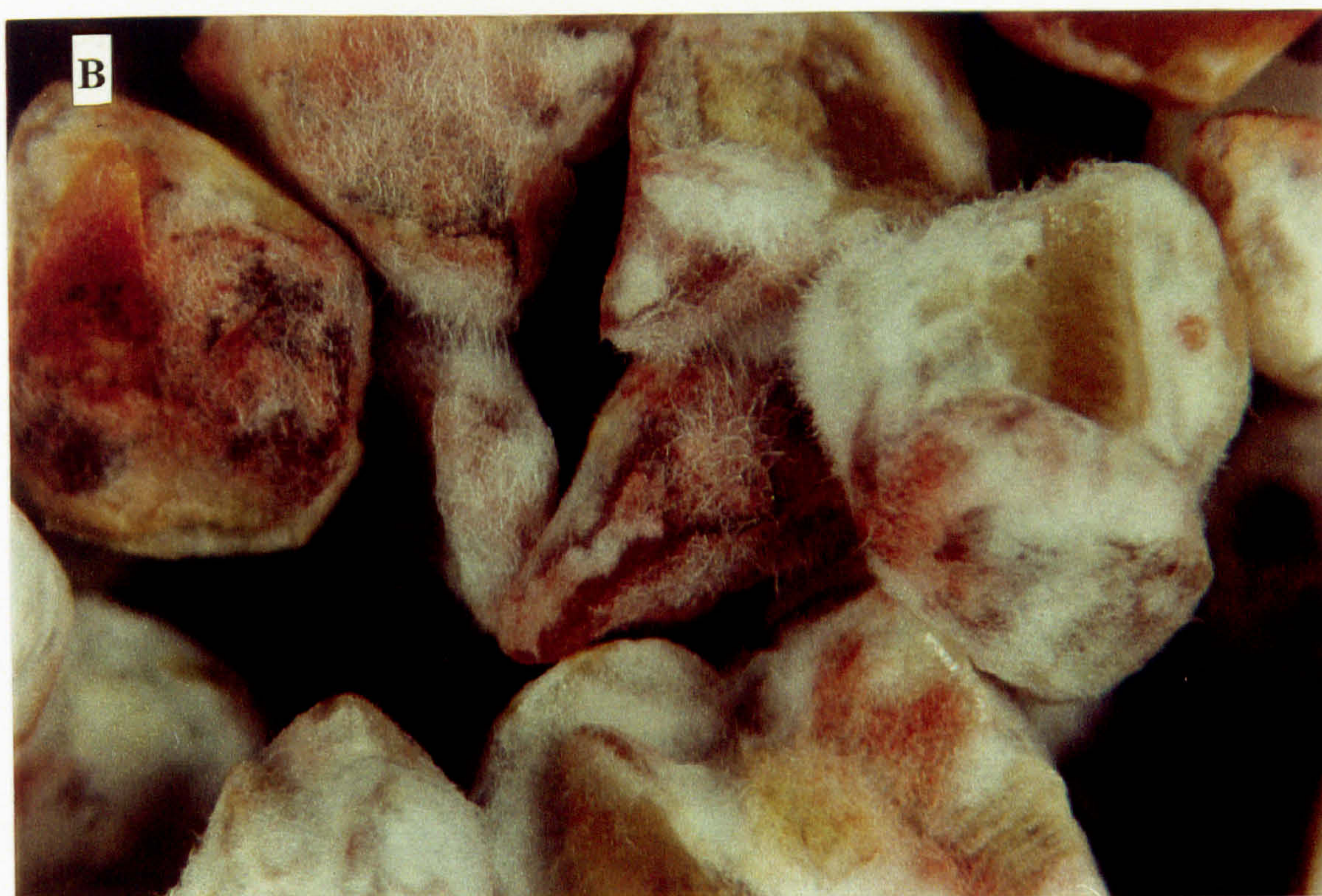


Plate 4.8. Pre-grown bulgar wheat inoculum. A – appearance of inoculum, 2 weeks shaken incubation at 25°C. B – x20 magnification. Note fine mycelial cover and pigment formation.

4.4.3. Changes in culture media a_w .

Fig. 4.19. shows the temporal changes in culture media a_w during the course of the study. It is clear that, irrespective of initial a_w , there was a general upward drift of a_w until, by day 24, all treatments were effectively saturated (approx. 0.995 a_w).

4.4.4. Temporal development of metabolites.

Fig. 4.20. a – e. shows the temporal development of major metabolites over 24 days at each a_w level. It has been possible to keep the absorbance scale the same for each graph, to allow direct comparison. Main target metabolites are those shown eluting at 6.9 min, designated as metabolite 1; 7.3 min, designated as metabolite 2; and 8.9 min, designated as metabolite 3. Other metabolites shown are 7.4 min (the second metabolite in the shouldered peak described in Section 4.3.2.), 8.0 min and 7.1 min. Peak areas of metabolite 3 developed early in this study and remained at essentially uniform levels after day 7, increasing slightly in some treatments. Metabolite 2 appeared after approx. day 7 and then remained relatively constant, only increasing in the 0.97 a_w treatment. Levels of these metabolites were virtually unchanged by manipulation of a_w level. In contrast, metabolite 1 continued to accumulate up to day 24, and responded positively to lowered a_w level.

Additional metabolites were produced in the driest treatment (0.97 a_w), and also for the shaken treatment. The observation of additional metabolites for low a_w conditions is in keeping with earlier studies in this project. The shaken treatment produced a

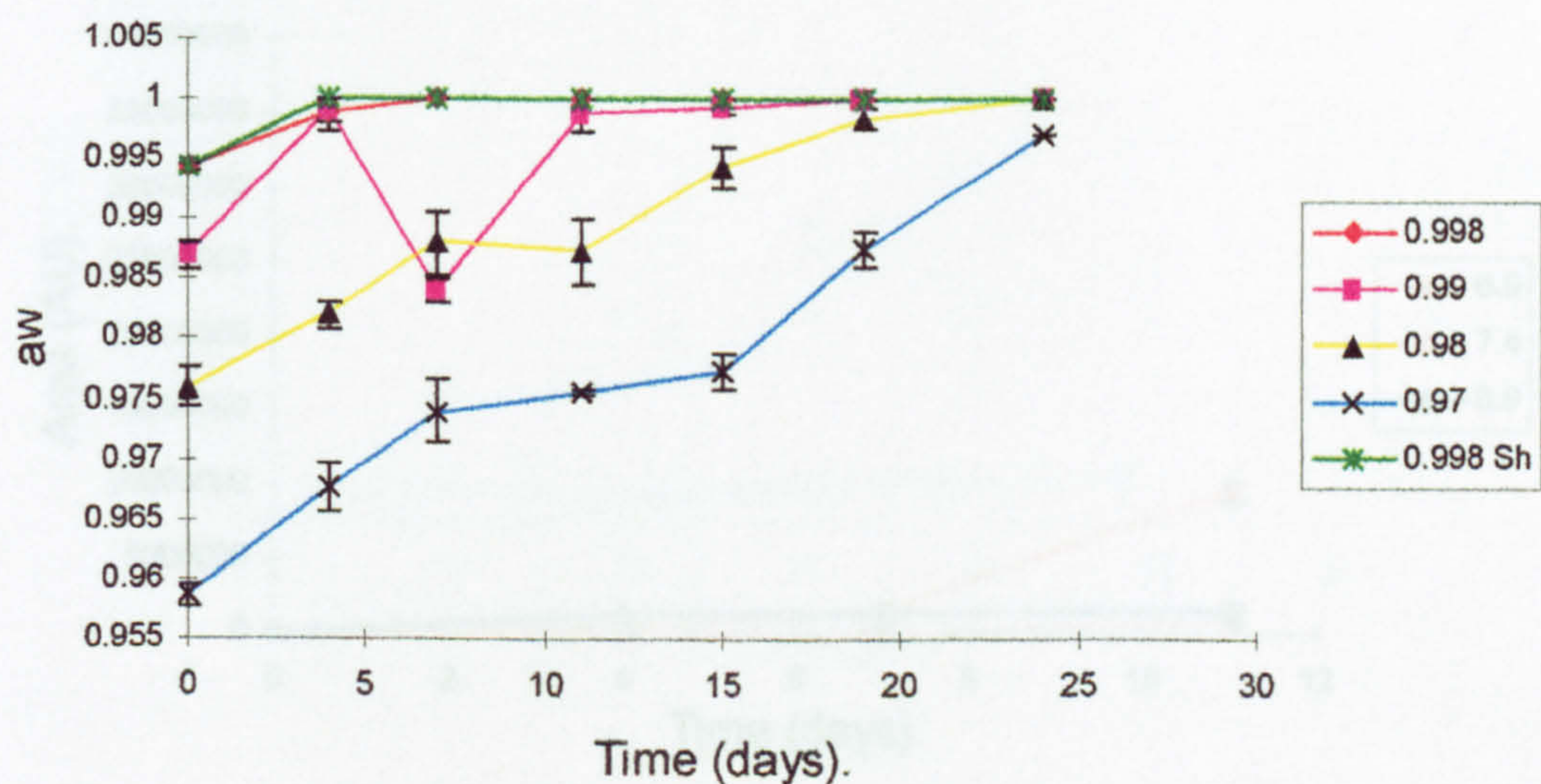
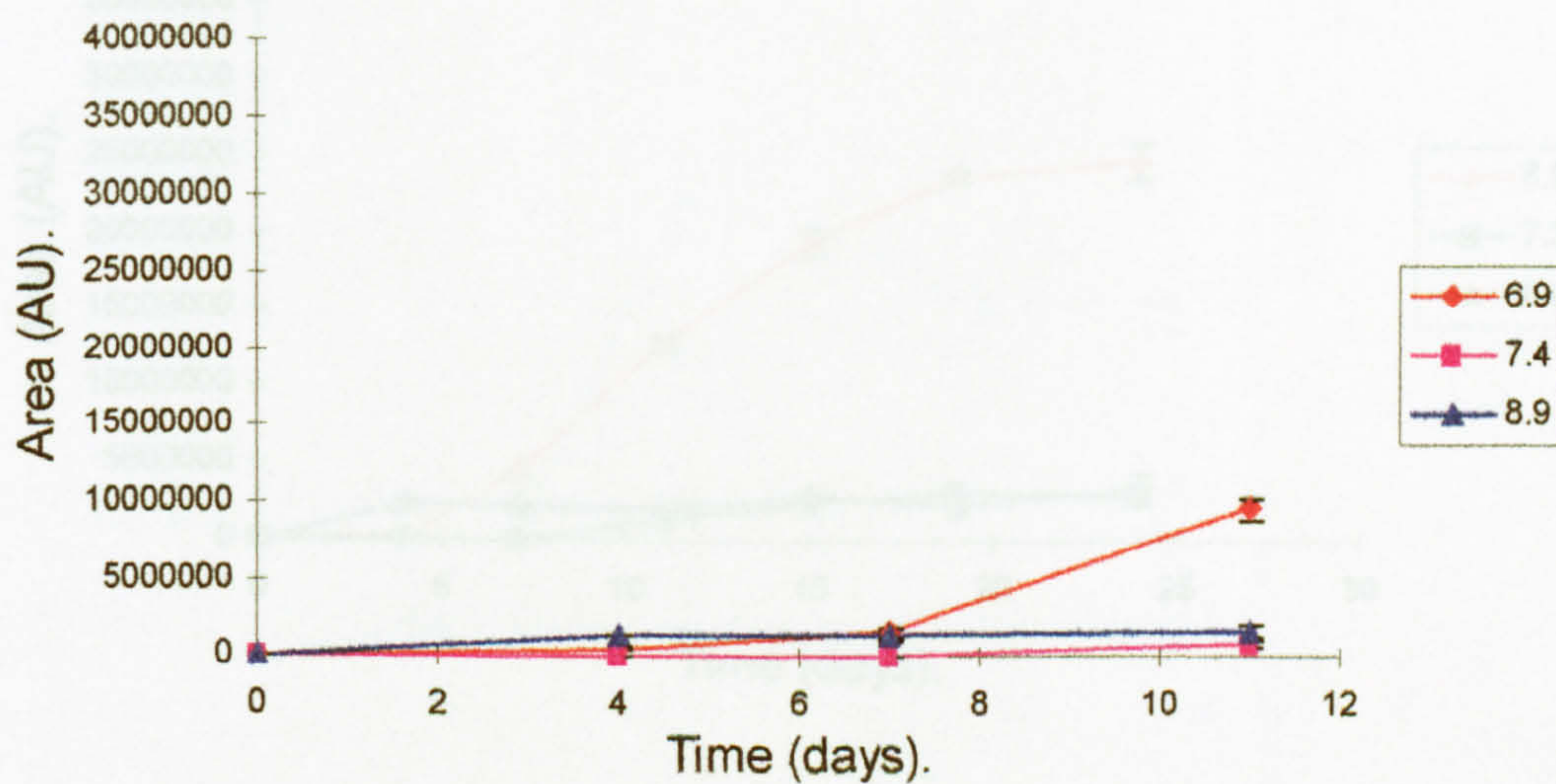


Figure 4.19. Graph of temporal changes in a_w of culture medium (bulgar wheat) during growth of *E. nigrum*. Bulgar wheat set to initial a_w levels of 0.998, 0.990, 0.980 and 0.970.

0.998 Sh = Treatment set to 0.998 a_w and incubated in reciprocating shaker. All other treatments incubated statically. Error bars are standard error of mean.

a. 0.998 a_w .



b. 0.990 a_w .

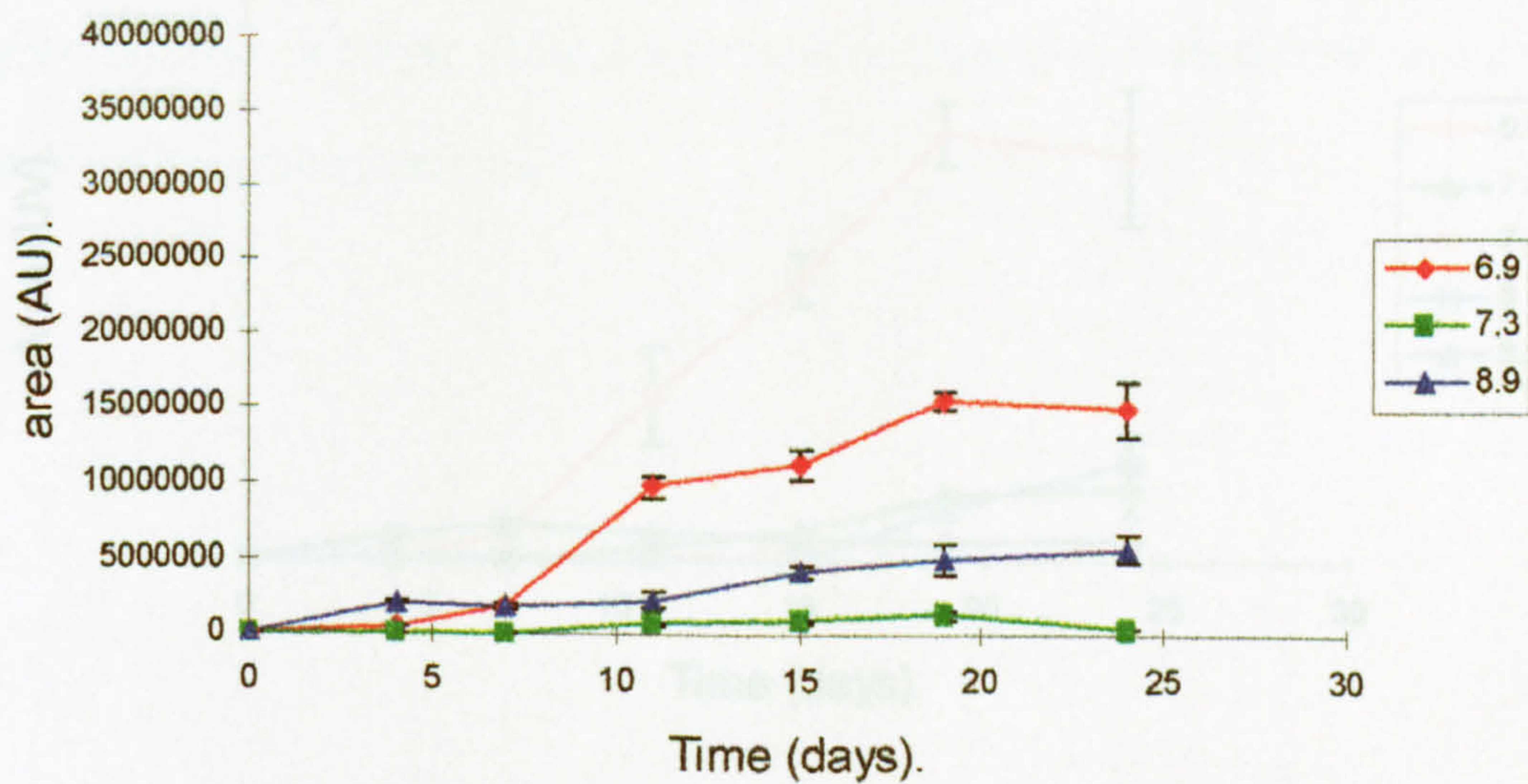
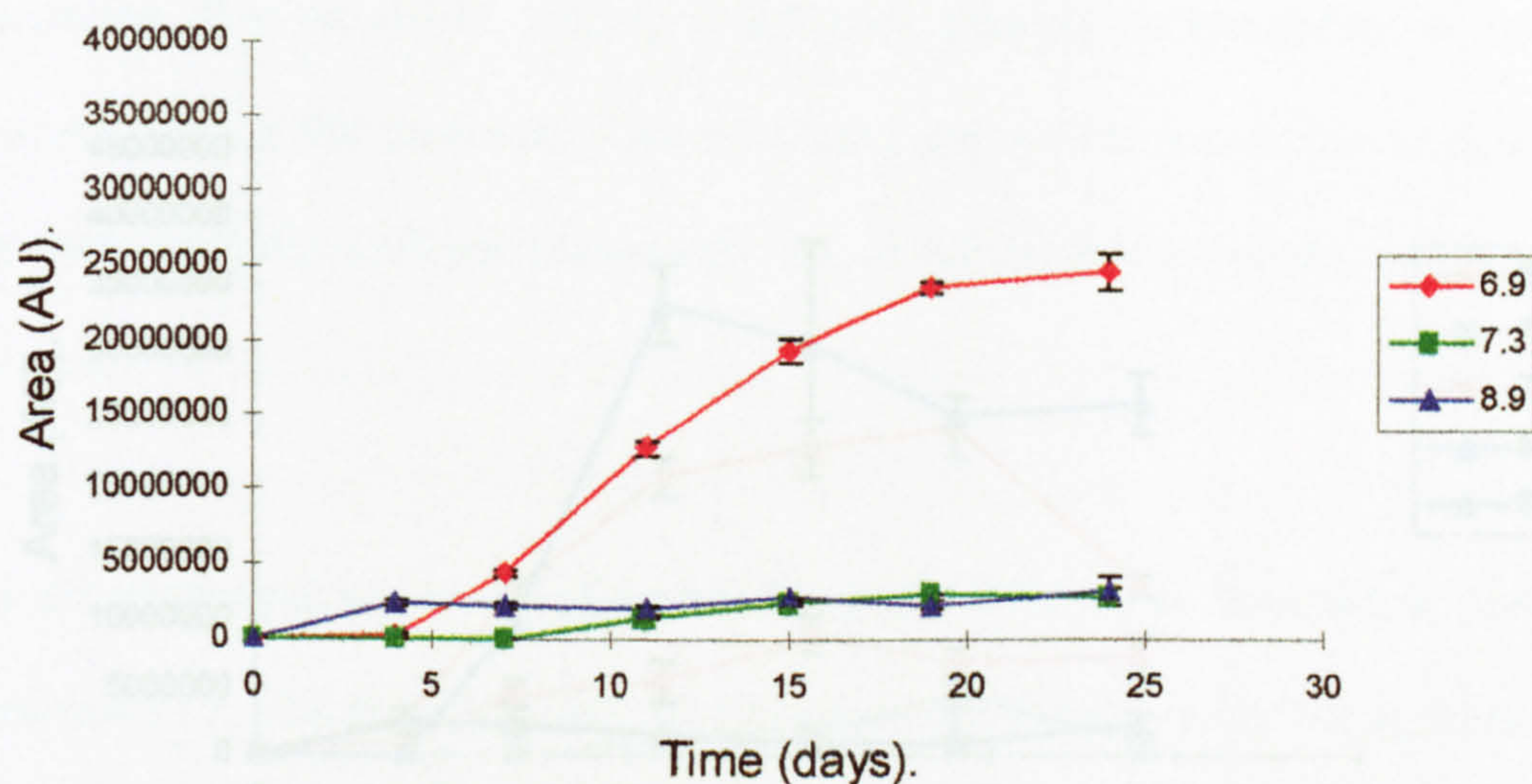


Figure 4.20 (cont.) Graph showing temporal development of metabolites for *E. nigrum*

growing on bulgar wheat. Metabolites are measured in absorption units and identified by elution time (min).

c. 0.980 a_w



d. 0.970 a_w .

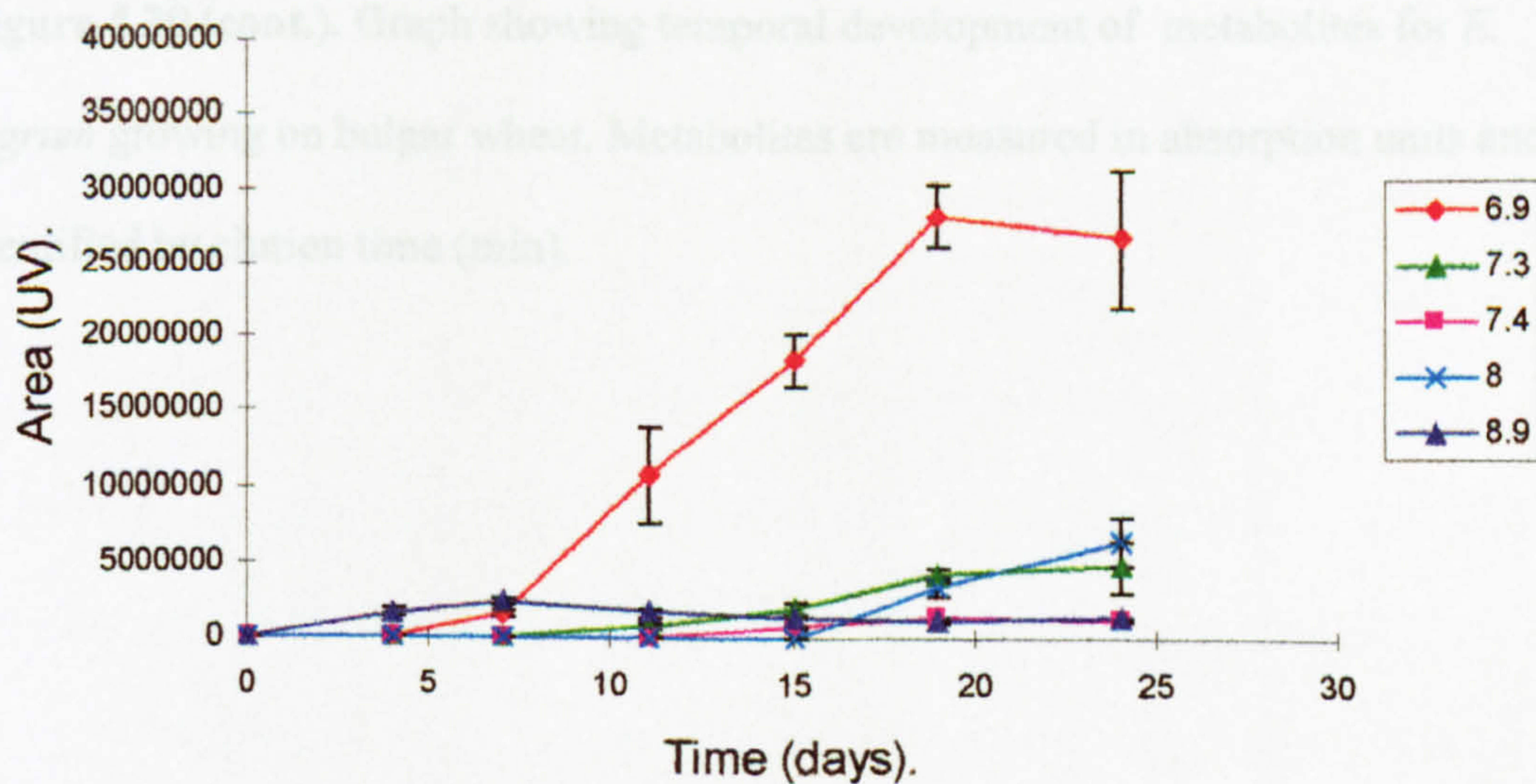


Figure 4.20 (cont.). Graph showing temporal development of metabolites for *E. nigrum* growing on bulgar wheat. Metabolites are measured in absorption units and identified by elution time (min).

e. 0.998 a_w shaken treatment.

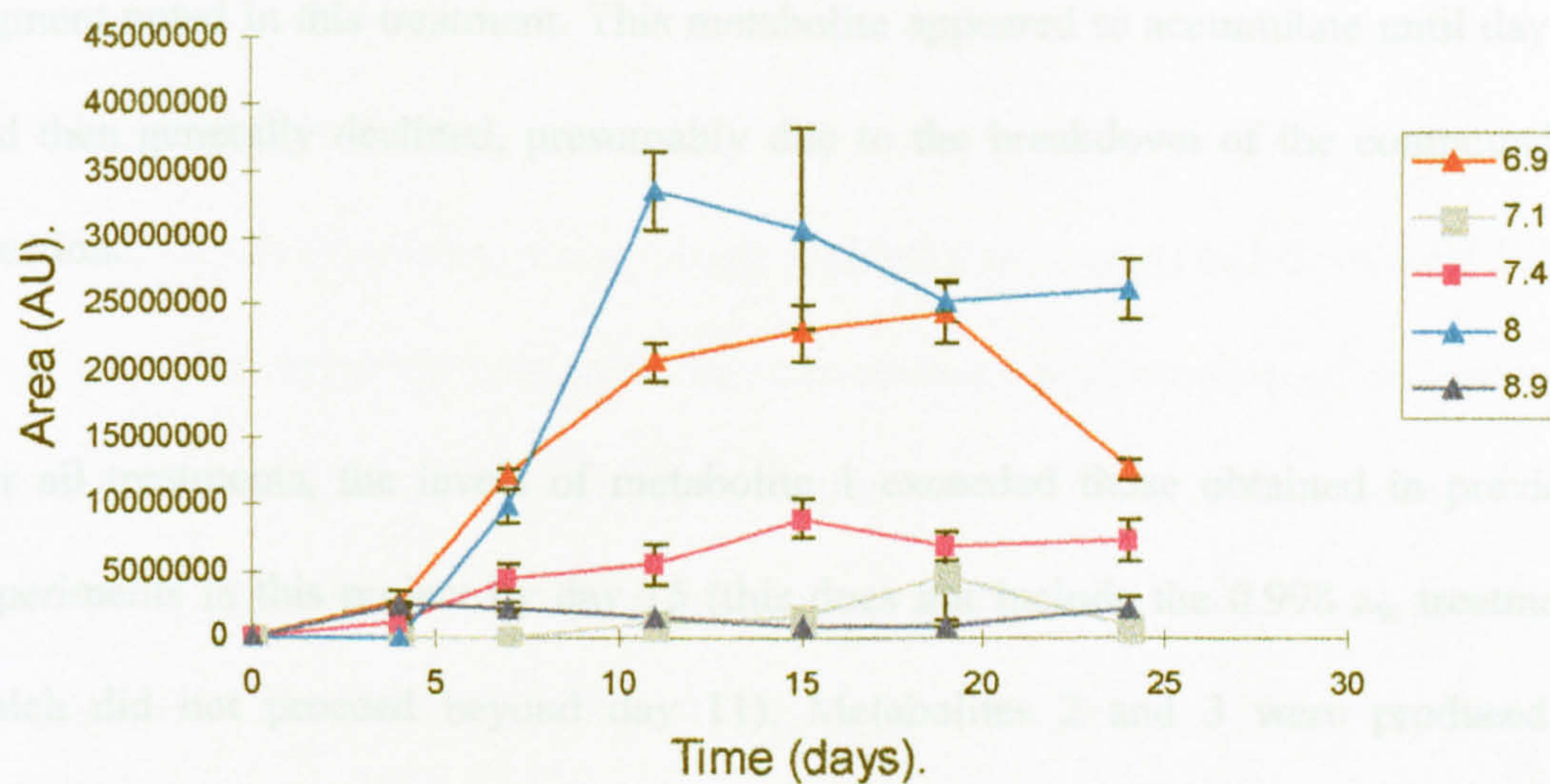


Figure 4.20 (cont.). Graph showing temporal development of metabolites for *E.*

nigrum growing on bulgar wheat. Metabolites are measured in absorption units and identified by elution time (min).

markedly different pattern of metabolites, characterised mainly by significant production of a metabolite eluting at 8.0 min, perhaps corresponding to the red pigment noted in this treatment. This metabolite appeared to accumulate until day 10 and then generally declined, presumably due to the breakdown of the compound in question.

For all treatments, the levels of metabolite 1 exceeded those obtained in previous experiments in this project by day 15 (this does not include the 0.998 a_w treatment, which did not proceed beyond day 11). Metabolites 2 and 3 were produced at comparable levels to those previously obtained.

4.5. EFFECTS OF AERATION AND AGITATION AT SMALL SCALE USING *E. NIGRUM* GROWN ON BULGAR WHEAT.

4.5.1. General observations.

At the end of this experiment the statically incubated treatments had developed 100 % cover of white mycelium with uniform yellow/orange pigmentation of the substrate particles. The aerated treatments were similar except for a small, occluded area immediately adjacent to the air entry points which was sparsely colonised and unpigmented. This effect appeared to be due to local drying at the air input point. The shaken treatments appeared as yellow/orange pigmented particles with no clear evidence of fungal hyphae. There was no evidence of production of a red pigment in these treatments, as had been noted in the previous experiments.

4.5.2. Secondary metabolite profiles.

Figure 4.21. shows secondary metabolite profiles (major and target metabolites) for *E. nigrum* growing on bulgar wheat under forced aeration, shaken and static incubation conditions. This shows production of metabolite 1 (6.9 min), metabolite 2 (7.36 min) and metabolite 3 (8.9 min). An additional metabolite was produced (static and aerated treatments only) at 7.9 min. Levels of metabolite 1 differed significantly between treatments. Highest levels were produced by static incubation, intermediate levels by aerated incubation and lowest levels by agitated incubation. Significant differences between these levels were confirmed by ANOVA and LSD analysis ($P = 0.05$) (see Table 4.4.). Patterns for metabolite 3 were similar although the levels obtained for

aerated and agitated treatments were not significantly different. Metabolite 2 was produced at uniform levels (for static and aerated treatments only).

Table 4.4. Statistical analysis for production of metabolite 1 by *E.nigrum* growing on bulgar wheat. Comparison of aerated, shaken and static incubation (P = 0.05).

Factor	F crit	F	LSD
Incubation type	5.14	40.56	3325280

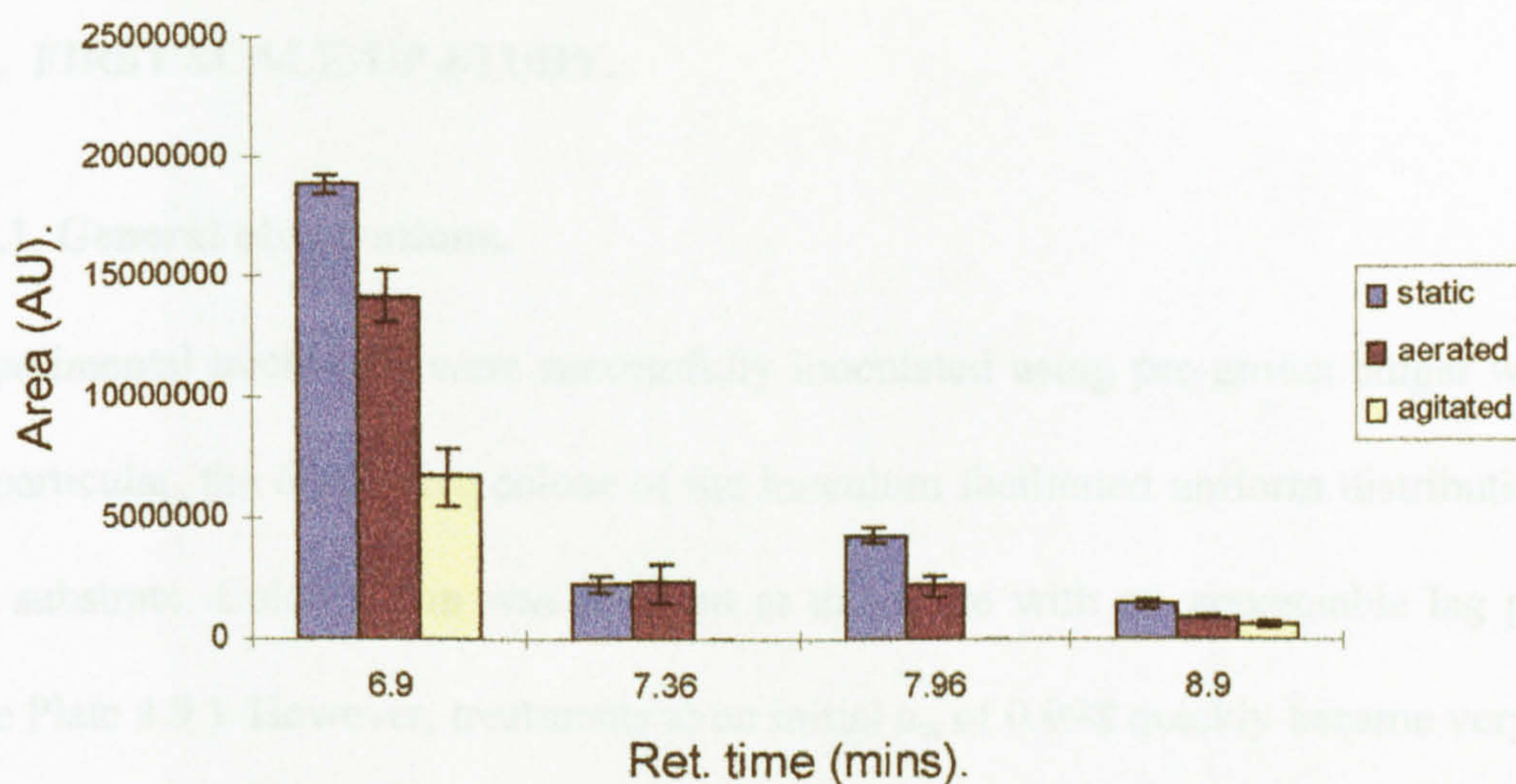


Figure 4.21. Secondary metabolite profile for *E. nigrum* grown on bulgar wheat under static, aerated and agitated incubation conditions. Metabolites are measured by peak area (absorption units) and identified by elution time (min). Error bars are standard error of mean.

4.6. FIRST SCALE-UP STUDY.

4.6.1. General observations.

Experimental treatments were successfully inoculated using pre-grown bulgar wheat. In particular, the distinctive colour of the inoculum facilitated uniform distribution in the substrate. Colonisation was efficient at this scale with no appreciable lag phase (see Plate 4.9). However, treatments at an initial a_w of 0.998 quickly became very wet and by day 3 appeared to be appreciably water-logged (see Plate 4.10). This amount of free water had an inhibitory effect on fungal growth and pigment development. Drier treatments also acquired significant quantities of free water which accumulated in the substrate and on the vessel walls above the substrate. All bottles became noticeably warm during the course of the study, typically rising to a temperature of approx. 30°C.

Fungal colonisation and pigment production in successfully completed treatments were as seen previously for this fungus/substrate system. Plate 4.11 shows a longitudinal section through the centre of a 0.97 a_w treatment at the end of the study. This plate indicates that pigment production occurred throughout the entire substrate column.

4.6.2. Bacterial contamination.

Bacterial contamination was a significant problem in the highest a_w treatments, and caused the abandonment of the 0.998 a_w shaken study by day 9. Also, by the end of



Plate 4.9. Early growth of pre-grown bulgar wheat inoculum, 24 hrs. incubation, 25°C, 0.97 a_w .



Plate 4.10. Water accumulation in 0.998 a_w treatment. Level of water saturation is visible on right of bottle.



Plate 4.11. Longitudinal section through substrate “bed” at day 14 showing uniform pigment production.

the experiment the 0.998 a_w static treatments demonstrated some degree of off-odour, which could be attributed to bacterial contamination. The contaminant was isolated from these treatments by direct plating of substrate particles on nutrient agar (NA) plates. The isolate was capable of producing confluent lawns on agar plates overnight at 25°C. It exhibited a dull “waxy” growth morphology, and gram staining revealed gram positive rods. The contaminant was presumptively identified as a *Bacillus* species (see Plate 4.12).

4.6.3. Metabolite profiles.

Metabolite profiles (major and target metabolites) for *E. nigrum* grown on bulgar wheat in 500 cm³ Duran bottles are shown in Figure 4.22. (a) shows results for static incubation and (b) shows results for shaken incubation. Figure 4.23. shows comparative results. This study produced levels of metabolite 1 (6.9 min), metabolite 2 (7.35 min), metabolite 3 (8.9 min) and the metabolite 2 “twin” (7.37 min). Apparent differences in levels of metabolite 1 between treatments were shown to be not significant by ANOVA and LSD analysis (Table 4.5), with the exception of the 0.99 a_w result, where significantly more metabolite was produced for shaken incubation. Other target metabolites were produced at comparable levels between static and shaken treatments.

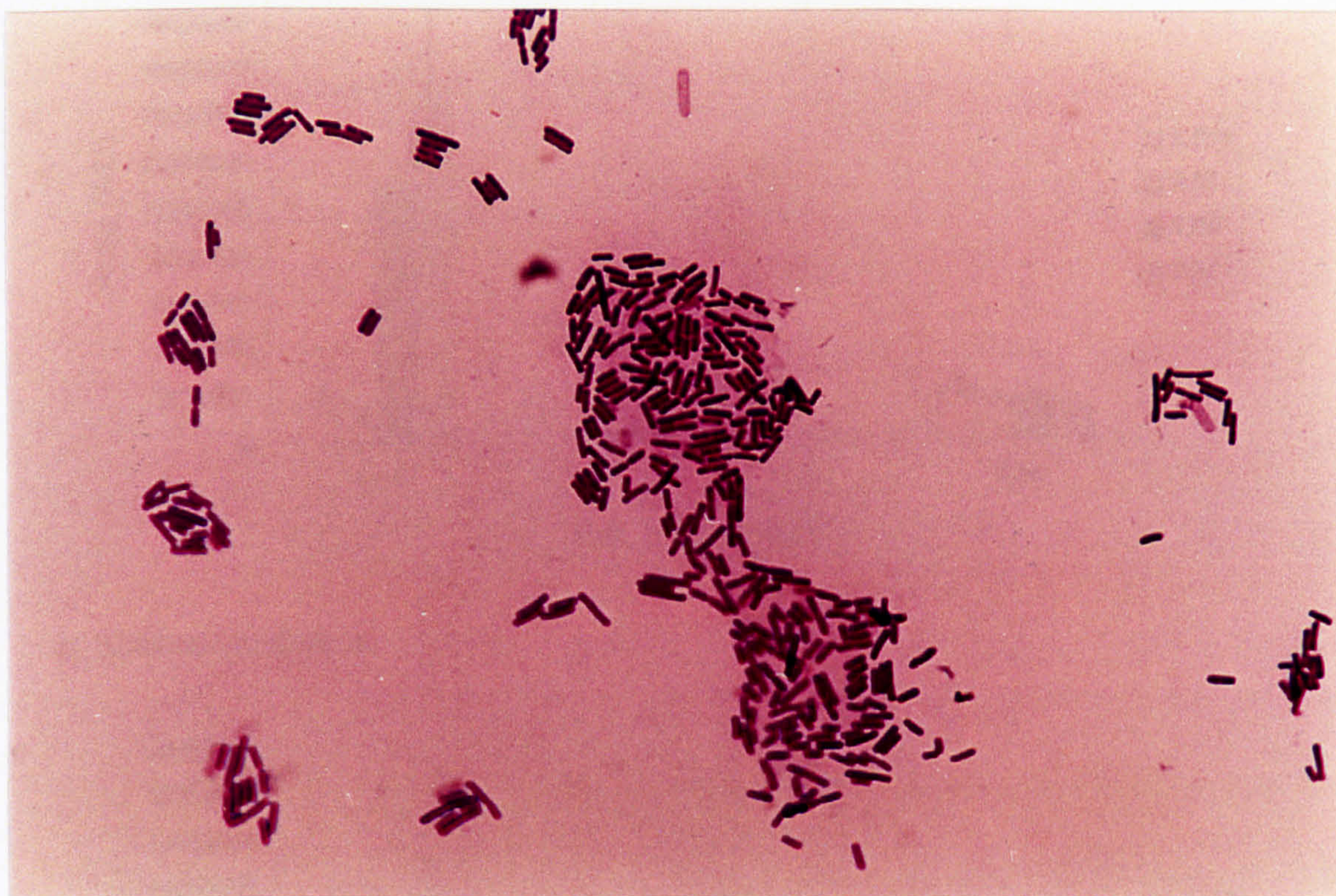
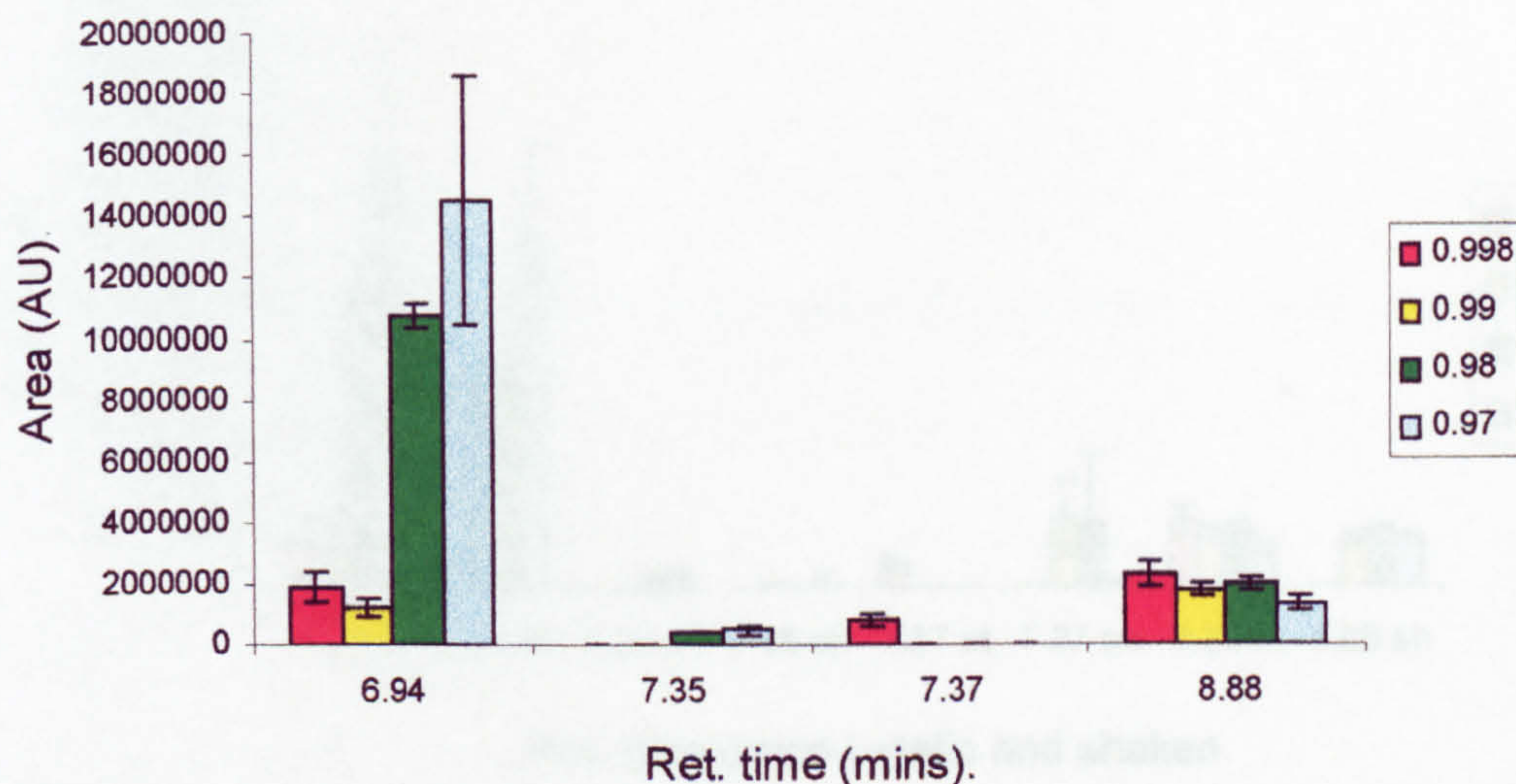


Plate 4.12. Bacterial contamination presumptively identified as a *Bacillus* species.

Gram stain of overnight culture, x1000 magnification, oil immersion.

a. Static incubation.



b. Shaken incubation.

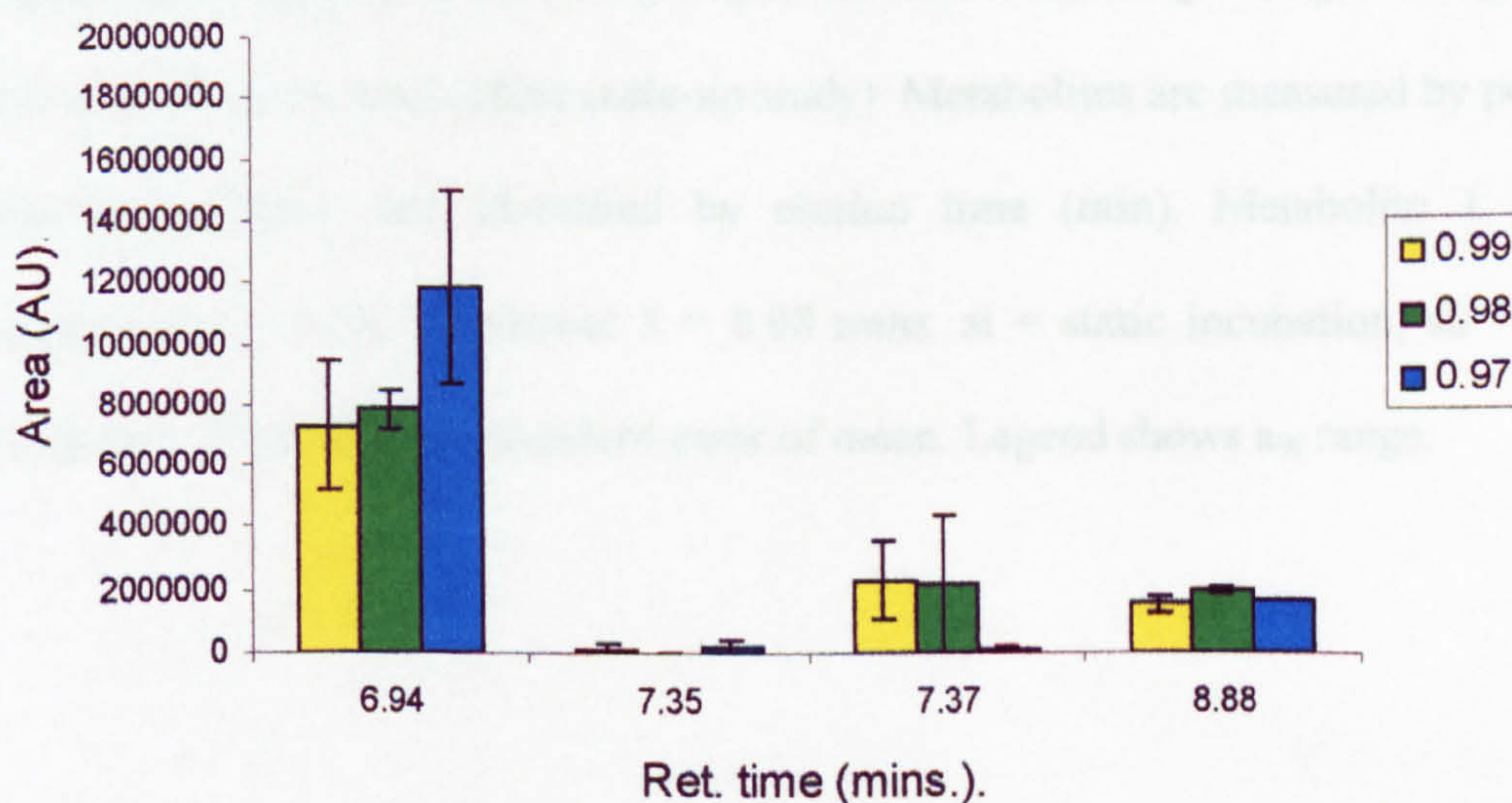


Figure 4.22. Metabolite profiles for *E. nigrum* growing on bulgar wheat in 500 ml Duran bottles over a range of a_w levels (first scale-up study). Metabolites are measured by peak area (absorption units) and identified by elution time (min). Metabolite 1 = 6.94, metabolite 2 = 7.35, metabolite 3 = 8.88 mins. Error bars are standard error of mean. Legend shows a_w range.

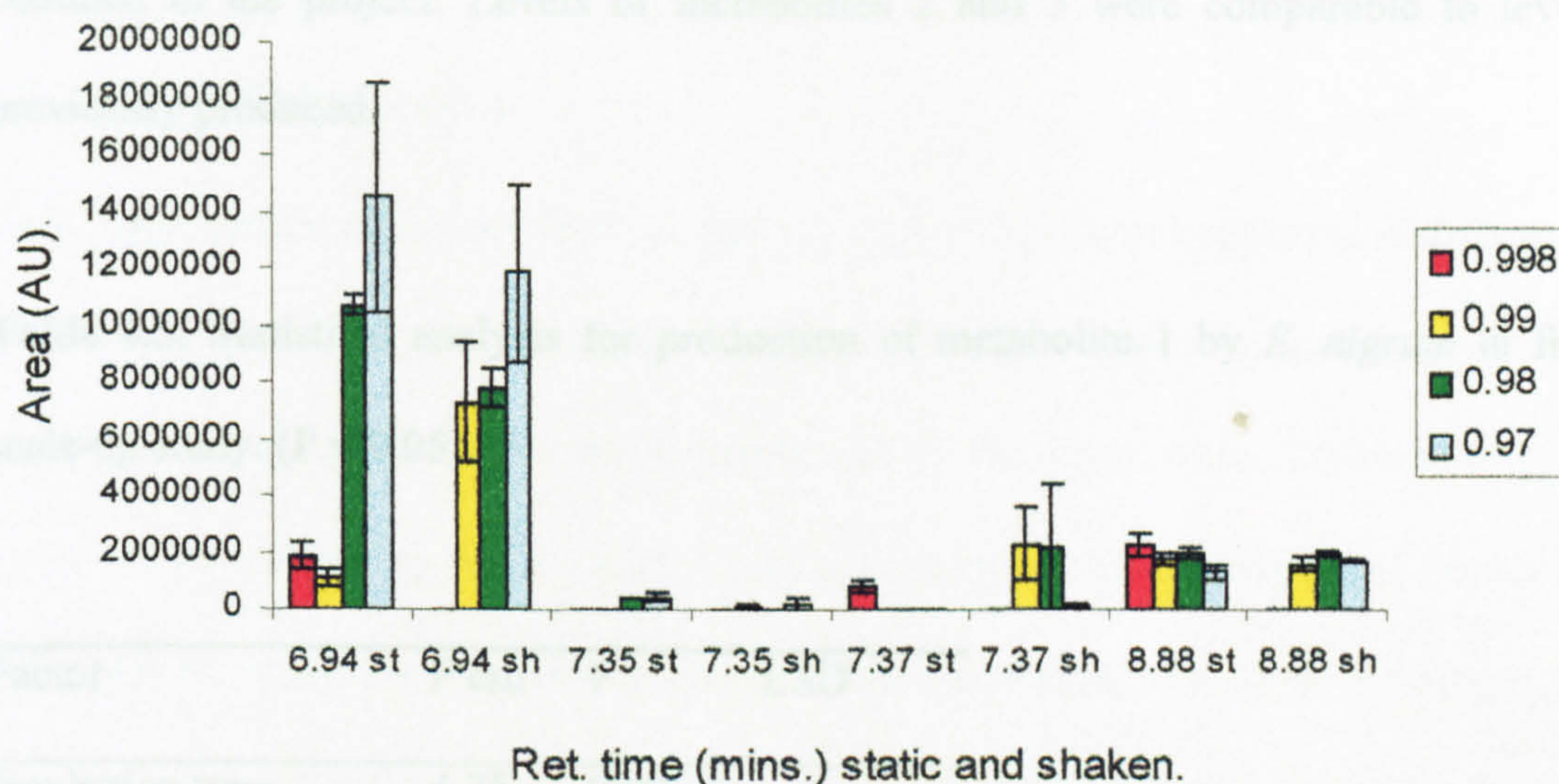


Figure 4.23. Comparative metabolite profiles for *E. nigrum* growing on bulgur wheat over a range of a_w levels (first scale-up study). Metabolites are measured by peak area (absorption units) and identified by elution time (min). Metabolite 1 = 6.94, metabolite 2 = 7.35, metabolite 3 = 8.88 mins. st = static incubation, sh = shaken incubation. Error bars are standard error of mean. Legend shows a_w range.

Levels of metabolite 1 produced in this study were in excess of levels previously obtained in the project. Levels of metabolites 2 and 3 were comparable to levels previously produced.

Table 4.5. Statistical analysis for production of metabolite 1 by *E. nigrum* in first scale-up study. (P = 0.05).

Factor	F crit	F	LSD
Incubation type	4.75	0.02	
a _w	3.89	12.09	
a _w x incubation type	3.99	3.88	5626153

4.7. FIRST LABORATORY SCALE FERMENTER STUDY.

4.7.1. General observations.

Early colonisation of the substrate at this scale (3 litre volume) appeared satisfactory, but ceased to develop beyond day 5. There was little pigment development and significant build-up of water in the substrate and on the vessel walls. This water began to fill the air exit tubing and the filter housing. In spite of this, the input and output air velocities remained constant throughout the study indicating that air flow through the substrate was relatively unconstrained. The temperature of the vessel wall was consistently significantly higher than ambient (typically approx. 30°C, measured using an external mercury thermometer). Removal of fermenter contents through the narrow neck of the vessel at the end of the experiment was a significant problem. It was necessary to continuously stir the material with a metal rod as it was shaken slowly through the opening.

4.7.2. Substrate mixing.

Separation of the vessels for rolling of the fermenter vessel proved to be manageable but inconvenient. This was a satisfactory method for mixing the inoculum into the comparatively large substrate bulk, but after only 24 hours the substrate was too tightly bound with fungal mycelium to be separated by this gentle type of mixing. Rolling was therefore abandoned at this point and the vessel was shaken manually once daily for approx. 1 min instead.

4.7.3. Bacterial contamination.

After 10 days incubation the fermenter contents were showing signs of bacterial contamination, identical to that encountered in previous studies. The substrate was excessively wet and taking on a softened appearance. There was also a distinct, unpleasant odour associated with the fermenter exit air.

4.7.4. Aeration.

Figure 4.24. shows results of a_w measurement of fermenter input and exit air between days 3 and 11. Since pure water was used in the humidifier bottle (i.e. not set to a lower a_w level by the use of glycerol), the theoretical maximum a_w of input air was 1.00. As expected, the results were lower than this, indicating a relatively inefficient uptake of water in the bubble column, and giving a relatively drier air stream entering the fermenter. In spite of some erratic results, the a_w of output air was generally higher than input air indicating that some water was being actively removed from the fermenter. However, the obvious build-up of water observed in the study indicates that the aeration regime used was inadequate to control moisture level.

4.7.5. Metabolite profile.

The secondary metabolite profile (major and target metabolites) obtained for this study is shown in Figure 4.25. This shows that the profile is highly atypical in comparison to those obtained in previous studies with the same organism and substrate. In particular, metabolite 1 (6.9 min.) was reduced to a trace peak with a high standard error. Metabolite 3 (8.9 min.) was produced at a comparable level to previous studies, but again with a high standard error. Metabolite 2 was absent from

this study. An additional peak (5.18 min.) was produced, which had occurred only once previously. This may have been associated with the bacterial contamination.

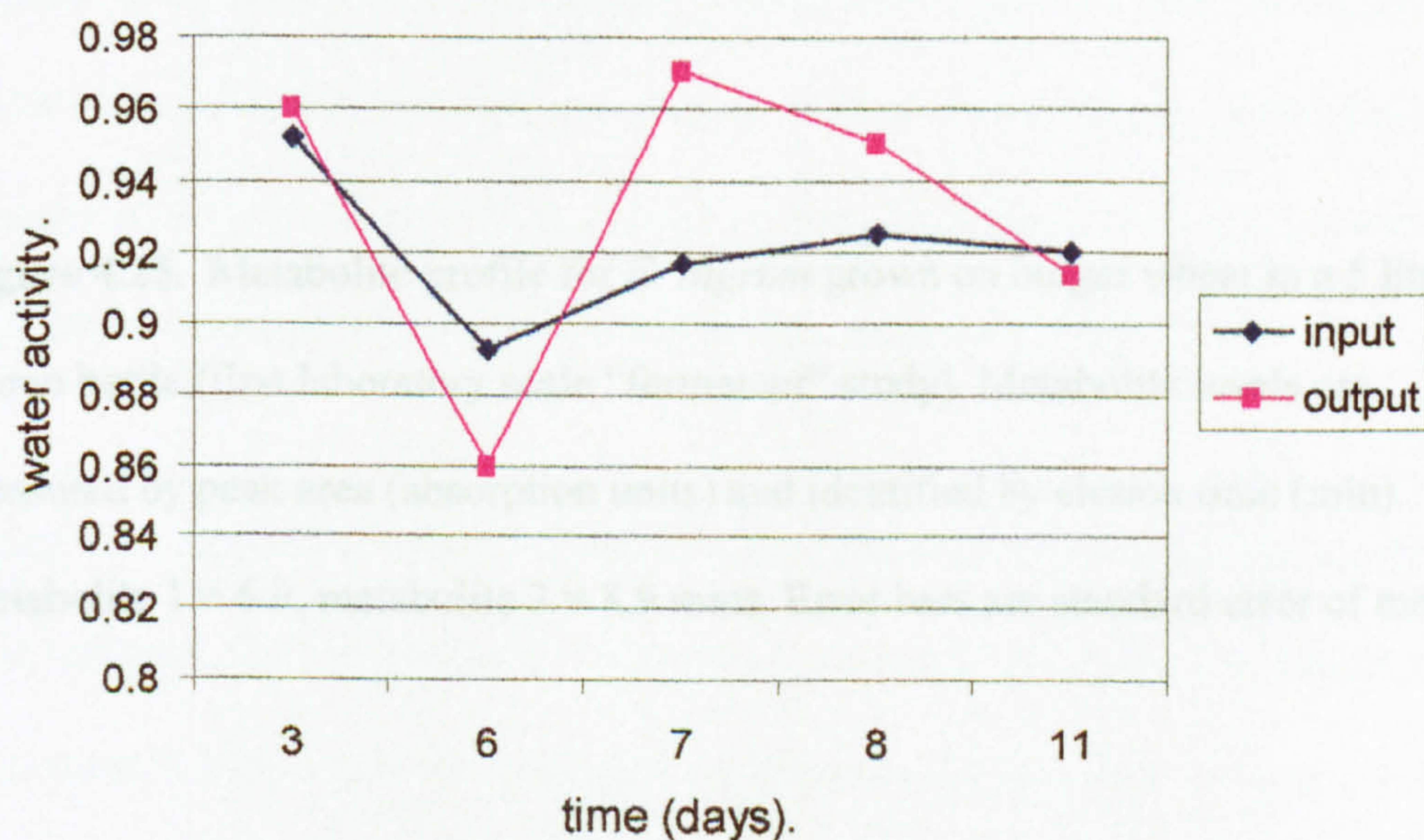


Figure 4.24. Graph of a_w of air entering fermenter (input air) and air leaving fermenter (output air) between days 3 and 11 for *E. nigrum* grown on bulgar wheat in a 5 litre Duran bottle.

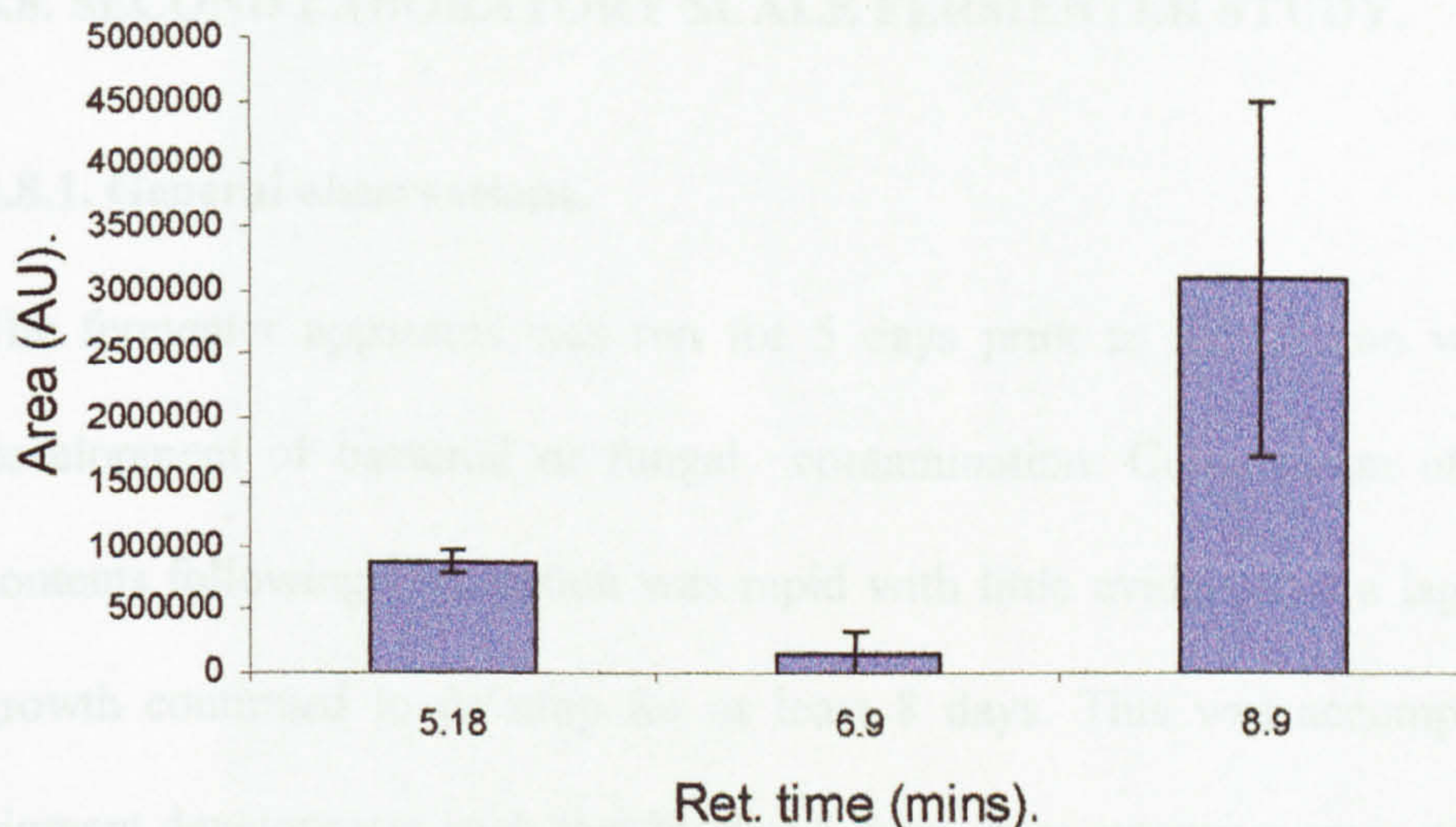


Figure 4.25. Metabolite profile for *E. nigrum* grown on bulgar wheat in a 5 litre Duran bottle (first laboratory scale “fermenter” study). Metabolite levels are measured by peak area (absorption units) and identified by elution time (min). Metabolite 1 = 6.9, metabolite 3 = 8.9 mins. Error bars are standard error of mean.

4.8.2. Bacterial contamination.

There was no clear evidence of bacterial contamination during the course of the study, although the material removed from the fermenter at completion showed slight signs of the same colour that had been very noticeable in previous studies. Most importantly,

4.8. SECOND LABORATORY SCALE FERMENTER STUDY.

4.8.1. General observations.

The fermenter apparatus was run for 5 days prior to inoculation with no sign of development of bacterial or fungal contamination. Colonisation of the fermenter contents following inoculation was rapid with little evidence of a lag phase. Fungal growth continued to develop for at least 8 days. This was accompanied by early pigment development such that by day 5 there were extensive areas of a deep orange colour. However, the absence of a mixing method in this study (particularly at the inoculation stage) had an obvious effect on the distribution of fungal growth - there were large areas that were not colonised satisfactorily (see Plate 4.13).

Figure 4.26. shows the fermenter and ambient temperatures recorded during the study. This indicates that the ambient temperature remained stable for the duration of the fermentation period and the fermenter temperature did not vary beyond ± 0.3 °C of ambient temperature. Emptying of the fermenter was relatively straightforward and it was possible to remove samples from the top, middle and bottom sections separately.

4.8.2. Bacterial contamination.

There was no clear evidence of bacterial contamination during the course of the study, although the material removed from the fermenter at completion showed slight signs of the same odour that had been very noticeable in previous studies. Most importantly,



Plate 4.13. Appearance of substrate at completion of 14 days incubation. Note absence of growth in bottom left section and pigment production in central area.

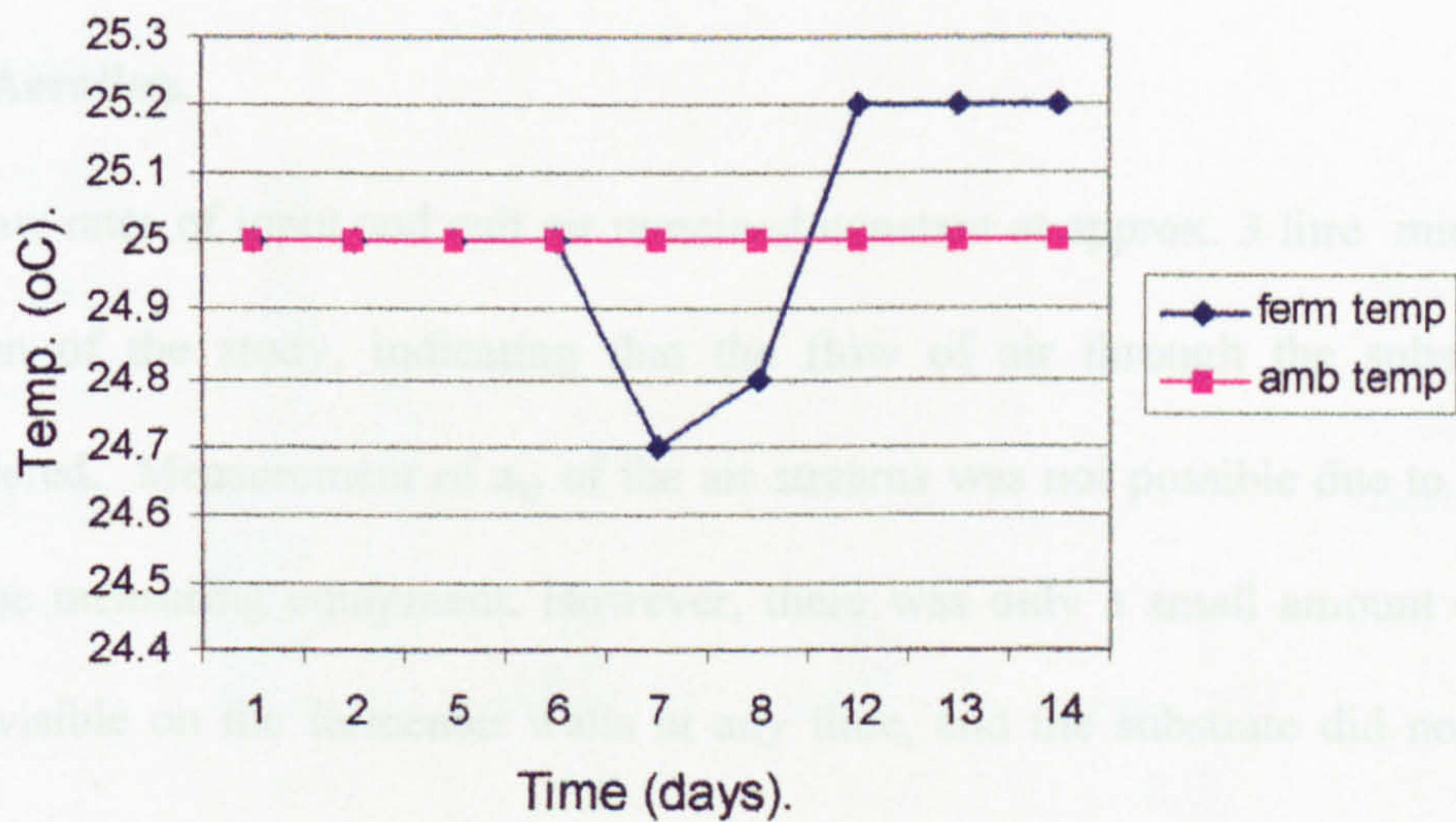


Figure 4.26. Fermenter and ambient temperatures for *E. nigrum* grown on bulgar wheat in a modified pilot scale submerged liquid fermenter (Bioengineering AG, Wald, Switzerland).

on culturing small samples of substrate on NA plates for 3 days at 30°C, no bacterial contamination was detected.

4.8.3. Aeration.

The flow rates of input and exit air remained constant at approx. 3 litre min⁻¹ for the duration of the study, indicating that the flow of air through the substrate was unhindered. Measurement of a_w of the air streams was not possible due to problems with the measuring equipment. However, there was only a small amount of excess water visible on the fermenter walls at any time, and the substrate did not become noticeably wet except for small areas towards the top of the substrate column. On emptying the fermenter at the end of the study, the substrate at the base nearest to the air entry points was drier than surrounding material, and this was one of the areas that did not become colonised by the fungus.

4.8.4. Metabolite profiles.

Secondary metabolite profiles (target and major metabolites) for *E. nigrum* grown on bulgar wheat in a modified laboratory scale fermenter are shown in Figure 4.27. The profiles show levels of metabolites for “top”, “middle”, “bottom” and overall average samples. Target metabolites obtained were metabolite 1 (6.9 min), metabolite 2 (7.3 min), metabolite 3 (8.9 min) and an additional metabolite at 8.02 min. Metabolite levels obtained in this study were encouraging. However there appeared to be significant variation in levels between the three sampling regions in the fermenter, in terms of increased levels in the “middle” section. This increase was shown to be

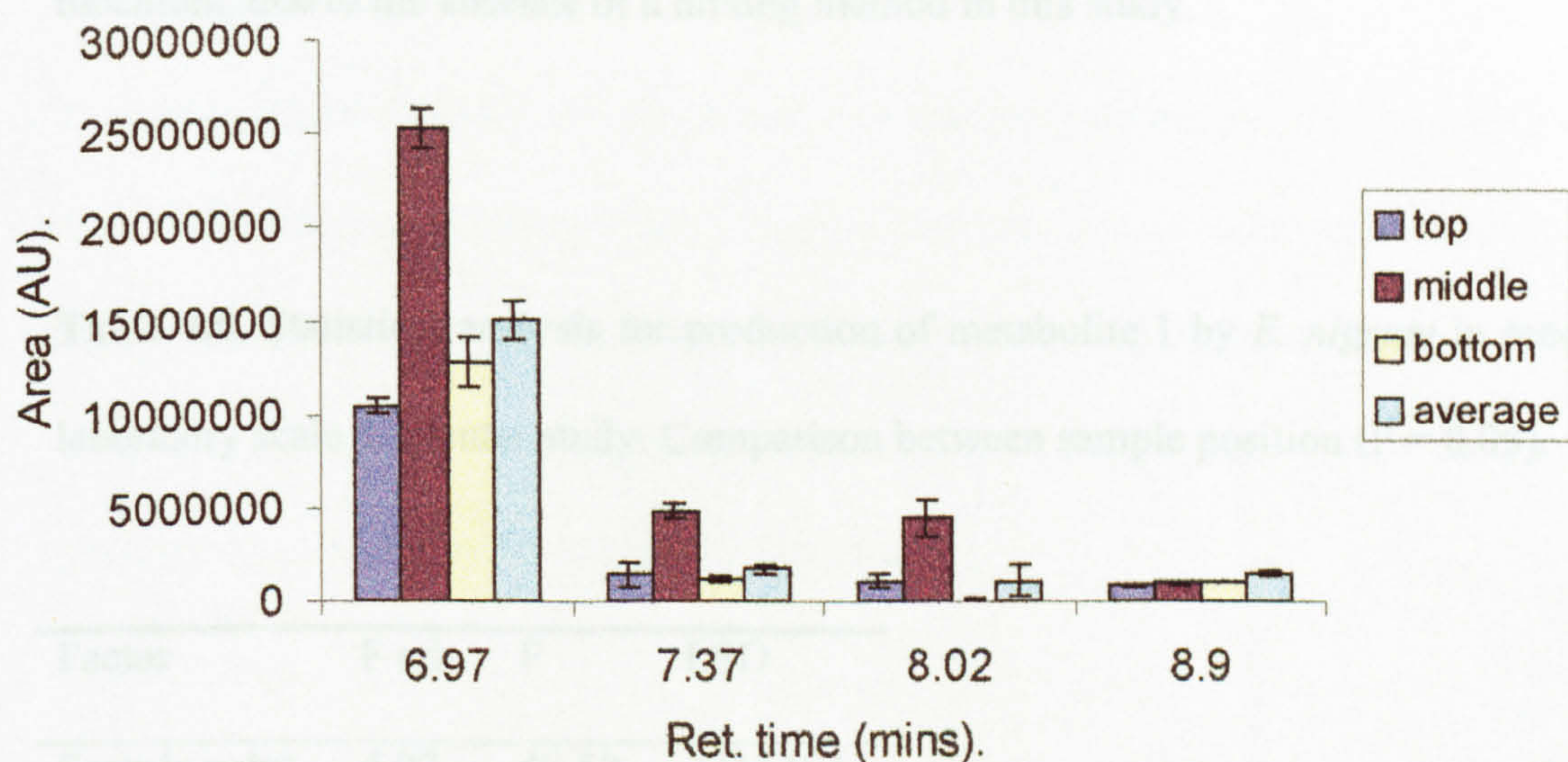


Figure 4.27. Metabolite profile for *E. nigrum* grown on bulgar wheat in a modified Bioengineering submerged liquid laboratory scale fermenter. Samples taken from top, middle and bottom of fermenter with additional sample representing overall average. Metabolites are measured by peak area (absorption units) and identified by elution time (min). Metabolite 1 = 6.97, metabolite 2 = 7.37, metabolite 3 = 8.9 mins. Error bars are standard error of mean. Legend indicates sample location.

significant, for metabolite 1, by ANOVA analysis ($P = 0.05$) (see Table 4.6.). This variation may have been a consequence of the poor distribution of the original inoculum, due to the absence of a mixing method in this study.

Table 4.6. Statistical analysis for production of metabolite 1 by *E. nigrum* in second laboratory scale fermenter study. Comparison between sample position ($P = 0.05$).

Factor	F crit	F	LSD
Sample point	4.07	40.50	3214767

4.9. THIRD LABORATORY SCALE FERMENTER STUDY.

4.9.1. General observations.

Arranging the air stream in the fermenter such that input air entered the headspace and output air exited at the base of the substrate bed appeared to work well since severe local drying was prevented. This arrangement continued to route the air stream efficiently through the substrate. Sinter glass components also performed satisfactorily, and did not become blocked in use.

Both runs showed rapid and uniform mycelial growth (see Plate 4.14) and very early pigment production such that by day 4 the substrate had developed a deep orange colour in both cases (see Plate 4.15). Run 1 was terminated at day 7 because of the well-developed pigment at this stage, and because no further changes appeared to be occurring. A similar pattern was shown in run 2, but this was continued until day 14.

4.9.2. Substrate mixing.

Inoculation with pre-grown bulgar wheat was followed by thorough mixing using the purpose-built impellers. This was a very efficient method for dispersing the inoculum; for both runs subsequent growth was rapid and uniform. Subsequent efforts to stir the substrate during growth in run 1 were less successful and were abandoned after a number of attempts. Stirring was not attempted in run 2 beyond mixing in the inoculum at the start of the fermentation.

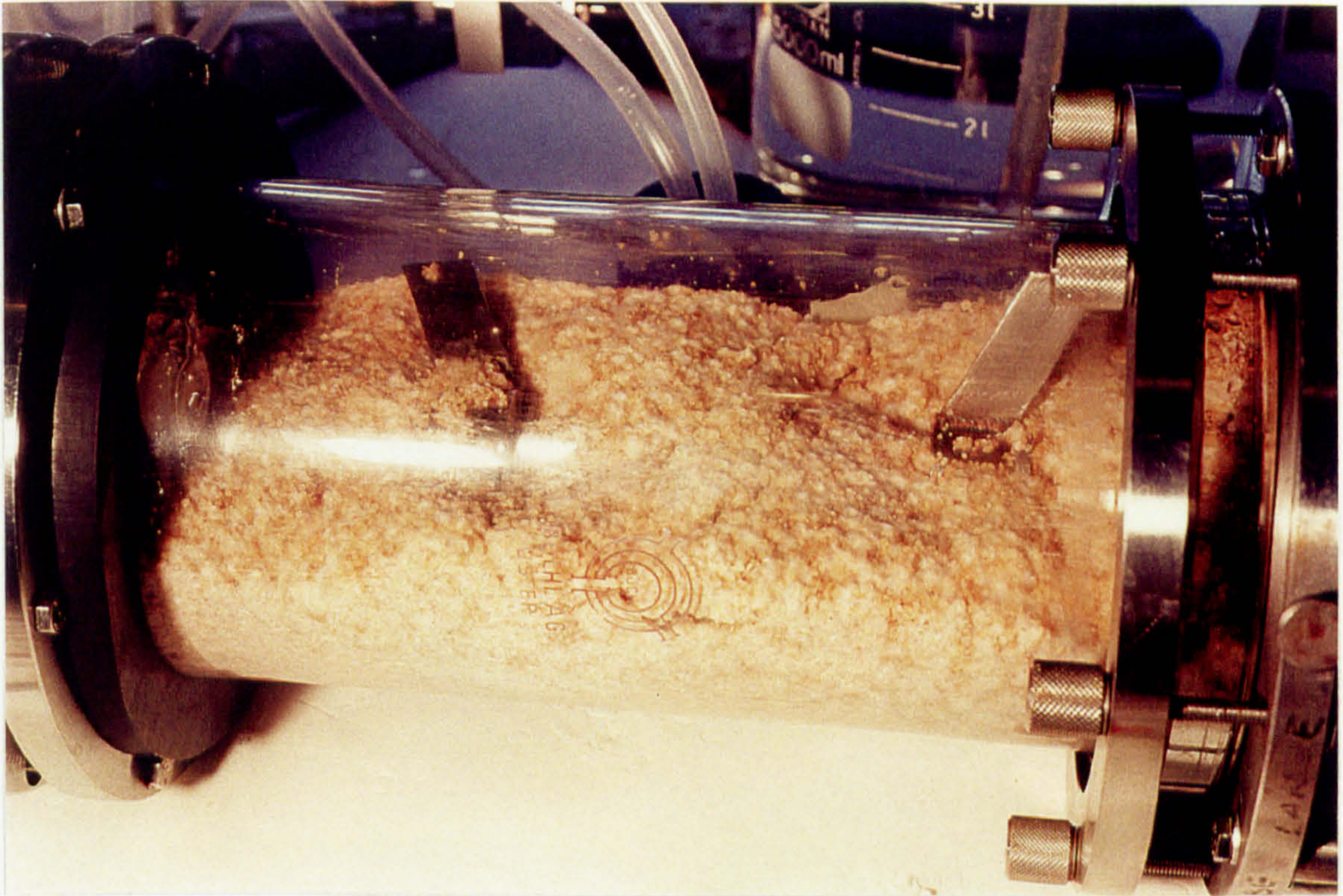


Plate 4.14. Run 1 at day 3, showing rapid and uniform colonisation. Note impeller blades protruding from substrate bed.

4.3.3. Bacterial concentration

Number of the time the run showed any form of bacterial contamination. There was



Plate 4.15. Run 2 at day 7, showing uniform pigment production. Note fermenter inclined at 45° angle.

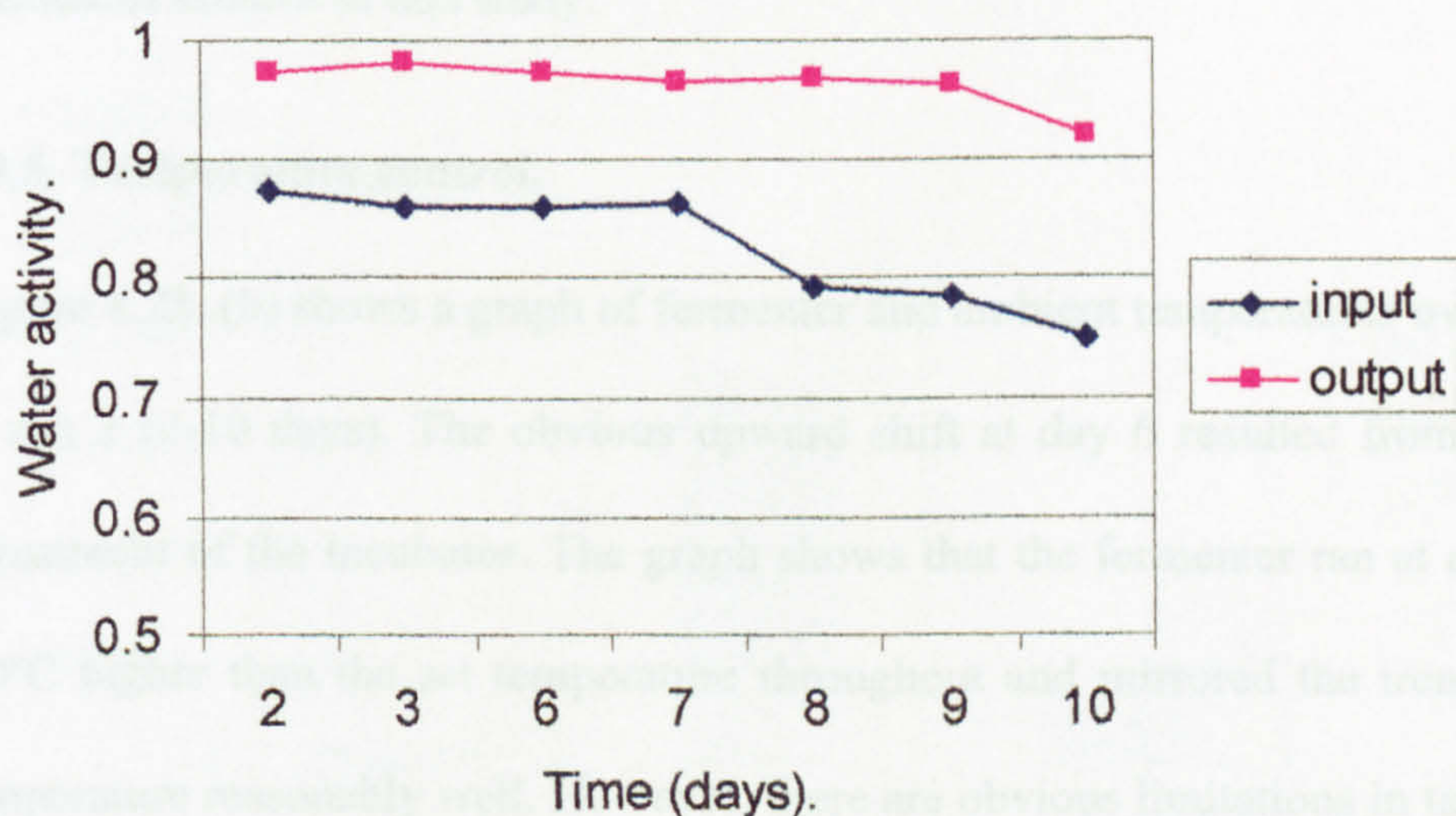
4.9.3. Bacterial contamination.

Neither of the fermenter runs showed any signs of bacterial contamination. There was no degradation of the substrate as had been noted in earlier studies, and no off-odours associated with the exit air. Run 2 was further protected against bacterial contamination by the addition of 0.1% chloramphenicol, and this did not seem to affect the outcome of the fermentation in any other way.

4.9.4. Aeration.

The flow rates of input and exit air remained constant at approx. 2 litre min⁻¹ for the duration of both studies, indicating that the flow of air through the substrate was relatively unhindered. Moisture accumulation was noted in run 1, but was removed efficiently by switching the air flow over to dry air (approx. 0.2 a_w). Dry air was passed through the fermenter on days 3 (2 hr), 4 (8 hr) and 5 (overnight). No obvious excess water accumulated during run 2. Figure 4.28 (a) shows the a_w of input and output air during the course of this fermentation (2-10 days). The graph shows the low a_w of input air resulting from the low a_w of the humidifier bottle, set at 0.90. As expected, the a_w of input air was lower than this value indicating less than maximum theoretical water uptake in the humidifier. Also noteworthy was the general decline in input air a_w over the course of the study. This probably resulted from the gradual loss of water from the humidifier, leading to an effective increase in glycerol concentration with time, and a corresponding fall in air a_w . The output air a_w remained uniformly higher than the value for input air throughout (approx 0.1 a_w differential) indicating

a. a_w of fermenter input and output air.



b. Fermenter and ambient temperatures.

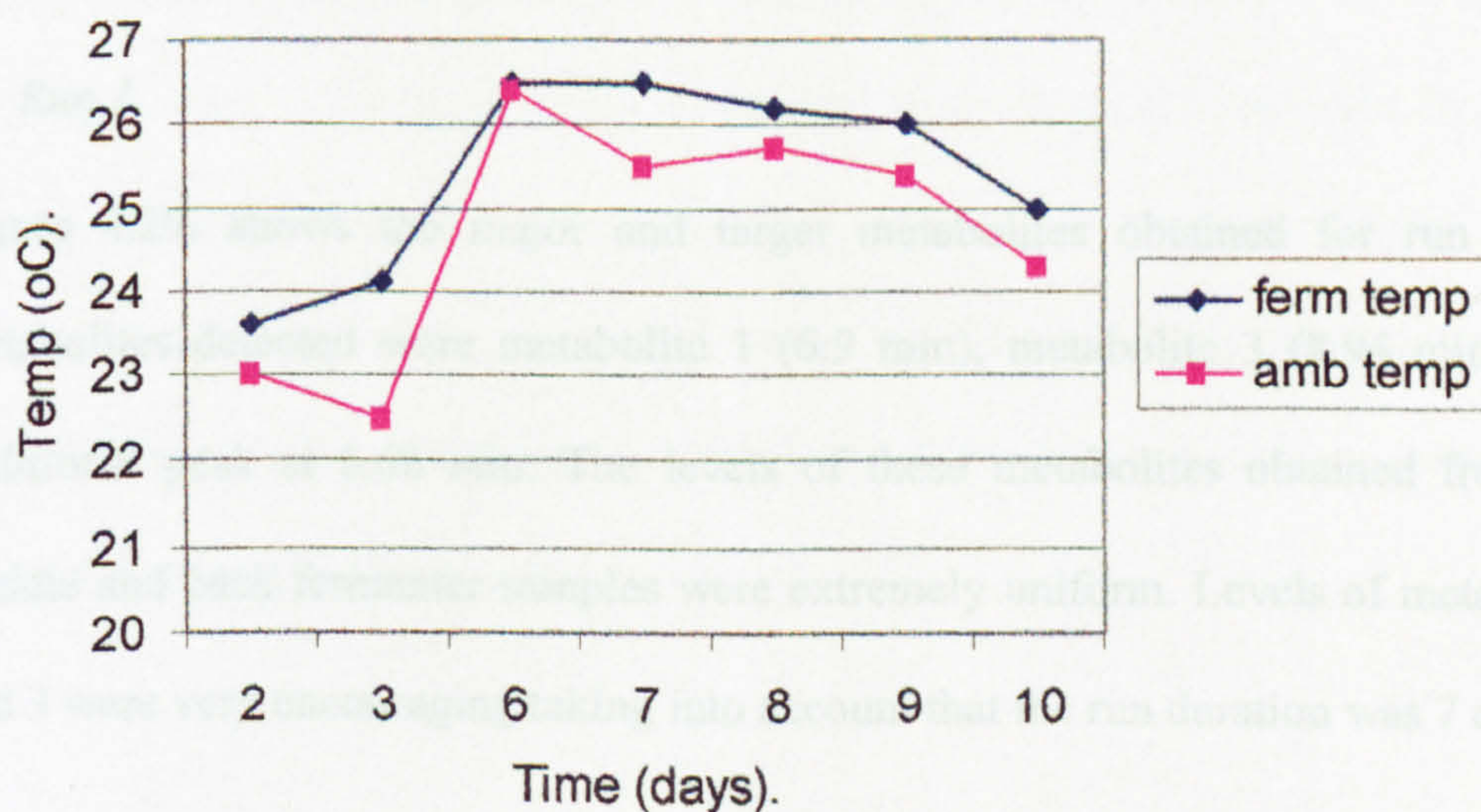


Figure 4.28. Fermentation conditions for *E. nigrum* grown on bulgar wheat in a modified Bioengineering submerged liquid laboratory scale fermenter oriented horizontally. Run 2.

that water was being removed from the fermenter in the air stream. This appeared to prevent obvious water accumulation and suggests that water content was under reasonable control in this study.

4.9.5. Temperature control.

Figure 4.28. (b) shows a graph of fermenter and ambient temperatures over the course of run 2 (2-10 days). The obvious upward shift at day 6 resulted from temperature adjustment of the incubator. The graph shows that the fermenter ran at approx. 0.5 – 1.0°C higher than the set temperature throughout and mirrored the trends in the set temperature reasonably well. However, there are obvious limitations in taking a single temperature measurement to represent the situation in the fermenter as a whole.

4.9.6. Metabolite profiles.

1. Run 1.

Figure 4.29. shows the major and target metabolites obtained for run 1. Main metabolites detected were metabolite 1 (6.9 min), metabolite 3 (8.94 min) and an additional peak at 6.66 min. The levels of these metabolites obtained from front, middle and back fermenter samples were extremely uniform. Levels of metabolites 1 and 3 were very encouraging taking into account that the run duration was 7 days.

2. Run 2.

Figure 4.30. shows the major and target metabolite profile obtained for run 2. Main metabolites detected were metabolite 1 (6.96 min), metabolite 2 (7.32 min),

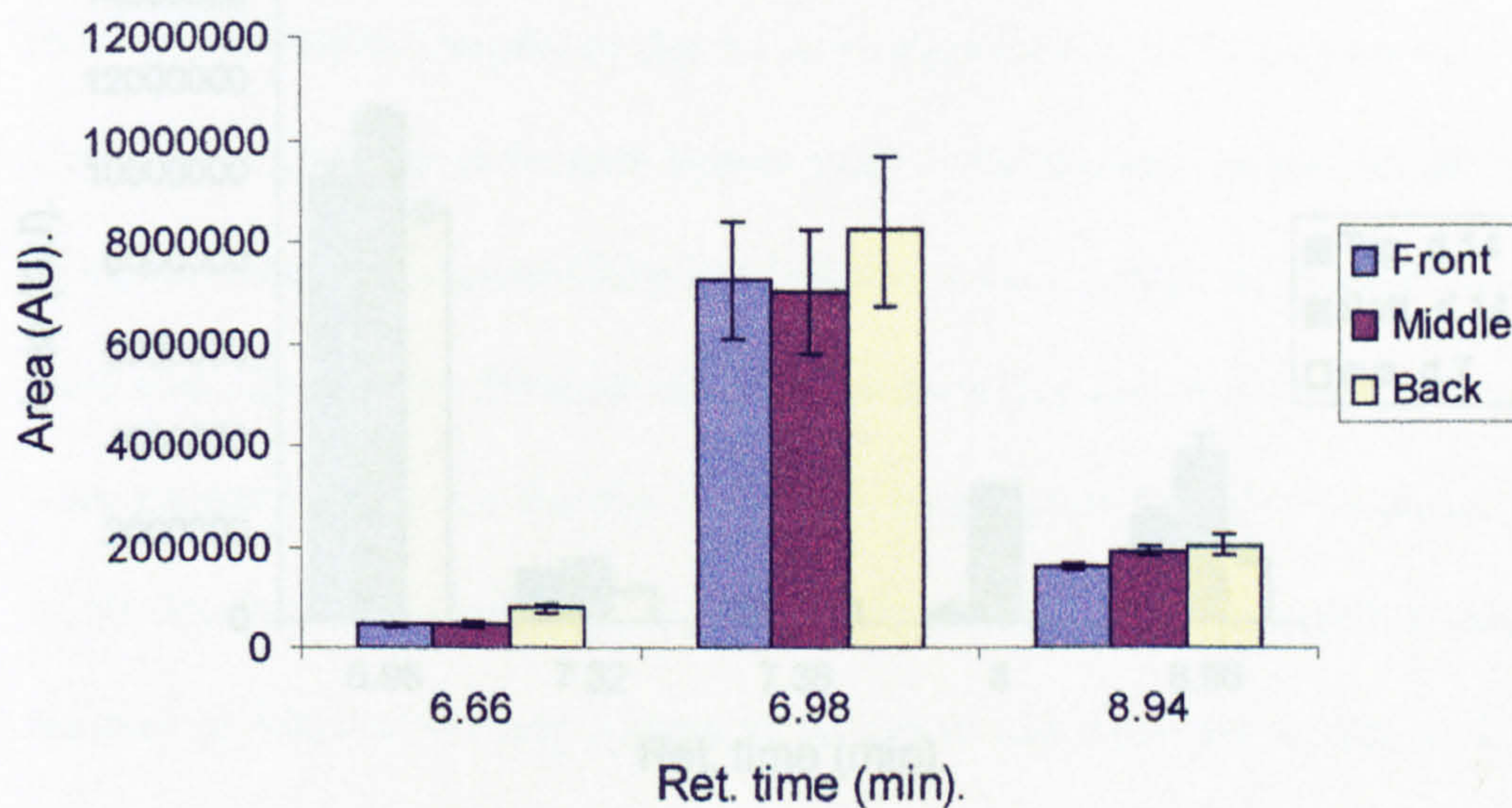


Figure 4.29. Metabolite profile for *E. nigrum* grown on bulgar wheat in a modified Bioengineering submerged liquid laboratory scale fermenter oriented horizontally. Run 1: Samples taken from front, middle and back of fermenter. Metabolites are measured by peak area (absorption units) and identified by elution time (min). Metabolite 1 = 6.98, metabolite 3 = 8.94 mins. Error bars are standard error of mean. Legend indicates sample location.

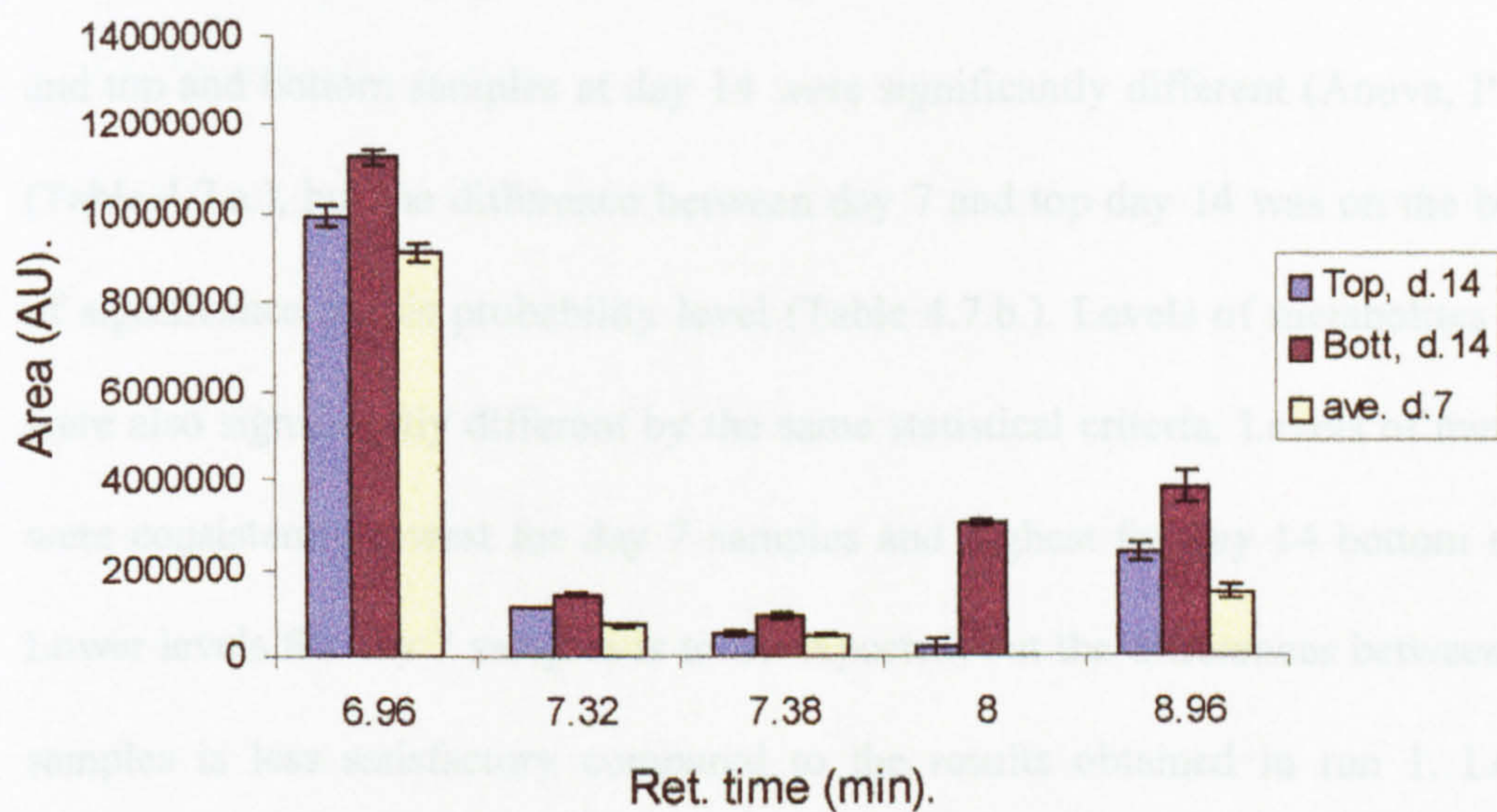


Figure 4.30. Metabolite profile for *E. nigrum* grown on bulgar wheat in a modified Bioengineering submerged liquid laboratory scale fermenter oriented horizontally. Run 2: Samples taken from top and bottom (bott.) of fermenter at day 14 and average (ave.) sample taken at day 7. Metabolites are measured by peak area (absorption units) and identified by elution time (min). Metabolite 1 = 6.96, metabolite 2 = 7.32, metabolite 3 = 8.96 mins. Error bars are standard error of mean. Legend indicates sample location and time.

metabolite 3 (8.96 min) and additional metabolites at 7.38 min (the “twin” of metabolite 2) and a metabolite eluting at 8.00 min, previously seen in the first modified Bioengineering fermenter study. Levels of metabolite 1 obtained at day 7, and top and bottom samples at day 14 were significantly different (Anova, $P = 0.05$) (Table 4.7.a.), but the difference between day 7 and top day 14 was on the boundary of significance at this probability level (Table 4.7.b.). Levels of metabolites 2 and 3 were also significantly different by the same statistical criteria. Levels of metabolites were consistently lowest for day 7 samples and highest for day 14 bottom samples. Lower levels for day 7 samples is to be expected, but the differences between day 14 samples is less satisfactory compared to the results obtained in run 1. Levels of metabolites 1 and 3 were comparable between run 1 and run 2 on the day 7 samples.

Table 4.7. a. Statistical analysis for metabolite 1 produced by *E. nigrum* in run 2. Comparison between all sample types. **b.** Comparison between day 7 and day 14 top sample ($P = 0.05$).

a.

Factor	F crit	F	LSD
Sample type	5.14	30.47	422292

b.

Factor	F crit	F	LSD
Time	7.70	7.80	820369

4.10. COMPARISON OF METABOLITE LEVELS FOR ALL STUDIES.

4.10.1. Metabolite profiles.

Overall comparisons of levels of metabolites 1, 2 and 3 in all studies were made. A more detailed examination was made of the results obtained at the lowest a_w levels (0.96 – 0.97) since these were relevant to the final fermenter studies. The highest values obtained in small scale studies, and the values obtained for bulgar wheat at small scale were compared with the first scale-up study and the two “horizontally orientated” fermenter studies. For the scale-up study, the values for statically incubated treatments were used and, for the two fermenter studies, the highest values obtained were used irrespective of sampling point in the fermenter.

4.10.2. Results at low a_w levels.

(i) *Metabolite 1.*

Results for metabolite 1 are shown in Figure 4.31. The highest level of this metabolite in small scale studies was for medium A. ANOVA and LSD analysis revealed that there were no significant differences between the first scale-up study, medium A and the second horizontal fermenter study. The first horizontal fermenter study produced significantly lower levels than the first scale-up study. The small scale bulgar wheat study produced significantly less metabolite than any other treatment (see Table 4.8.). For example, the second horizontal fermenter run produced an increase of approx.

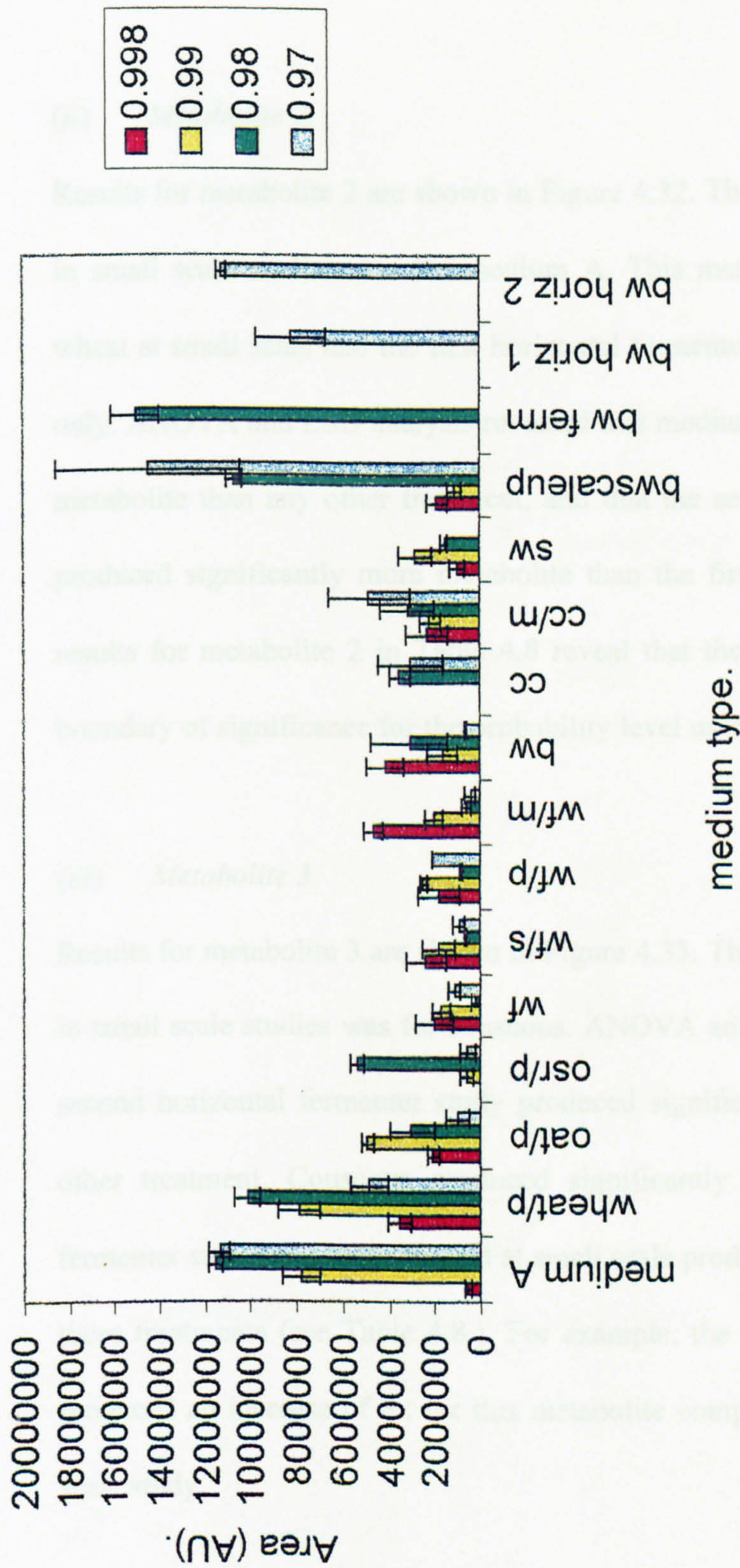


Figure 4.31. Comparison of levels of metabolite 1 measured by peak area (absorbance units) produced by *E. nigrum* for all studies. Error

bars are standard error of mean. Key to treatments: sw = shredded wheat, p = perlite, wf = wheat flakes, m = millet, bw = bulgar wheat, cc =

couscous, scaleup = first scale-up study, bw ferm = first modified Bioengineering fermenter study, bw horiz = horizontally orientated

fermenter studies. Fermenter studies carried out at one a_w level.

17x for this metabolite compared to the corresponding small scale study.

(ii) *Metabolite 2.*

Results for metabolite 2 are shown in Figure 4.32. The highest level of this metabolite in small scale studies was for medium A. This metabolite was absent from bulgar wheat at small scale and the first horizontal fermenter study, which ran for one week only. ANOVA and LSD analysis revealed that medium A produced significantly more metabolite than any other treatment, and that the second horizontal fermenter study produced significantly more metabolite than the first scale-up study. However, the results for metabolite 2 in Table 4.8 reveal that the differences in levels are at the boundary of significance for the probability level used.

(iii) *Metabolite 3.*

Results for metabolite 3 are shown in Figure 4.33. The highest level of this metabolite in small scale studies was for couscous. ANOVA and LSD analysis revealed that the second horizontal fermenter study produced significantly more metabolite than any other treatment. Couscous produced significantly more than the first horizontal fermenter study, and bulgar wheat at small scale produced significantly less than all of these treatments (see Table 4.8.). For example, the second horizontal fermenter run produced an increase of 3x for this metabolite compared to the corresponding small scale study.

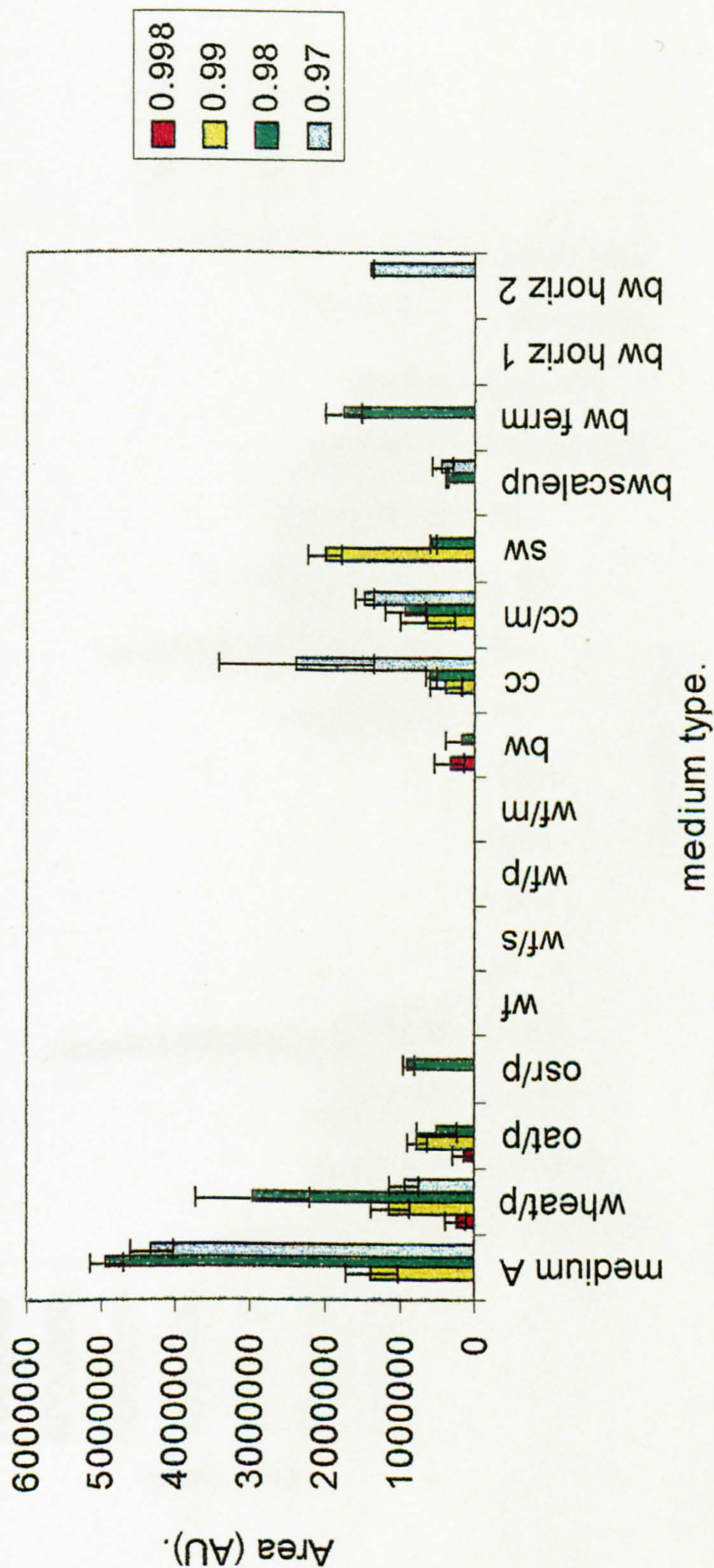


Figure 4.32. Comparison of levels of metabolite 2 measured by peak area (absorbance units) produced by *E. nigrum* for all studies. Error bars are standard error of mean. Key to treatments: sw = shredded wheat, p = perlite, wf = wheat flakes, m = millet, bw = bulgar wheat, cc = couscous, scaleup = first scale-up study, bw ferm = first modified Bioengineering fermenter study, bw horiz = horizontally orientated fermenter studies. Fermenter studies carried out at one a_w level.

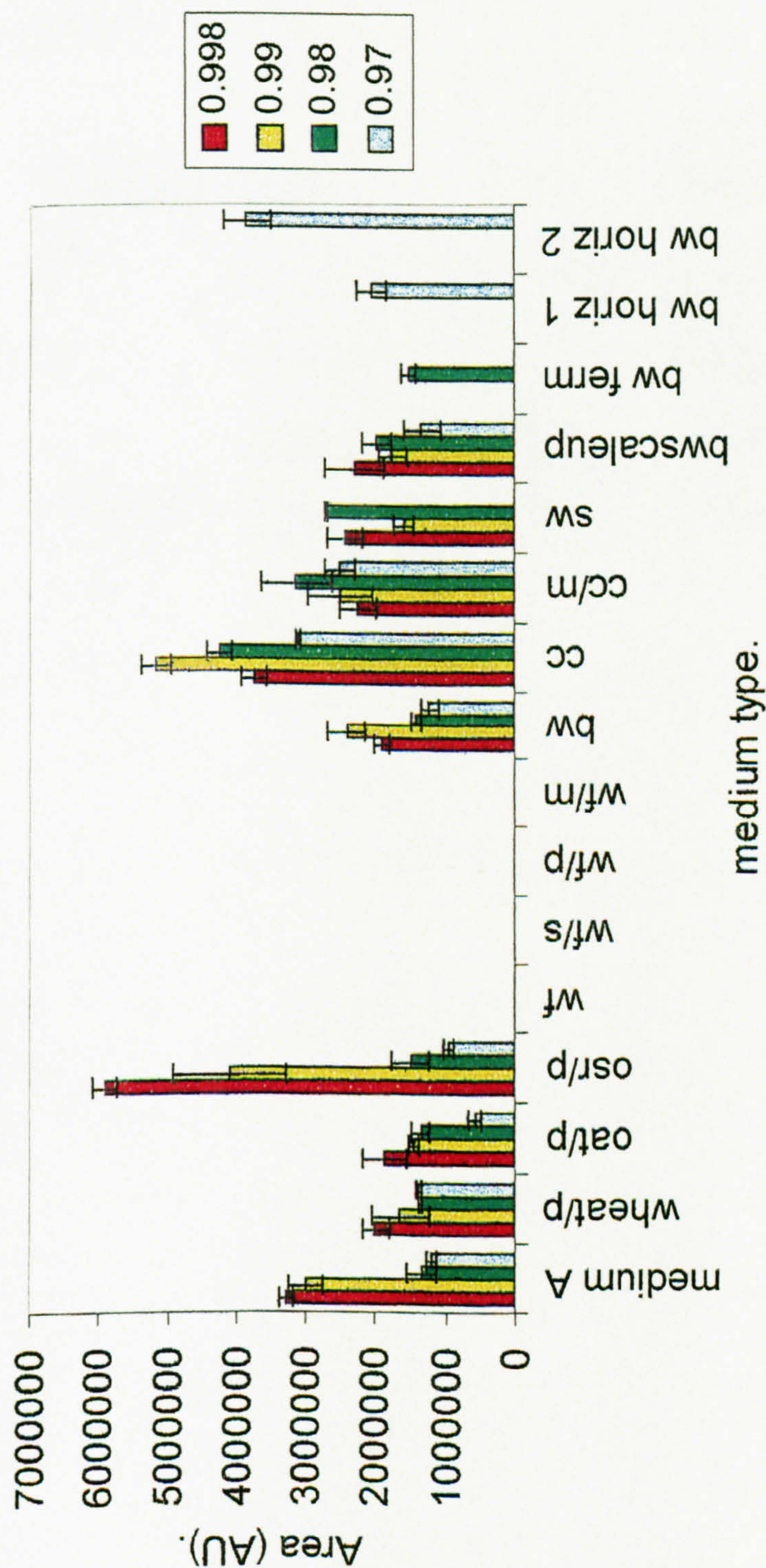


Figure 4.33. Comparison of levels of metabolite 3 measured by peak area (absorbance units) produced by *E. nigrum* for all studies. Error

bars are standard error of mean. Key to treatments: sw = shredded wheat, p = perlite, wf = wheat flakes, m = millet, bw = bulgar wheat, cc

= couscous, scaleup = first scale-up study, bw ferm = first modified Bioengineering fermenter study, bw horiz = horizontally orientated

fermenter studies. Fermenter studies carried out at one a_w level.

Table 4.8. Statistical analysis for production of metabolites 1, 2 and 3 by *E. nigrum* in all relevant studies (P = 0.05).

Metabolite	Factor	F crit	F	LSD
1	Study type	3.47	17.66	3964755
2	Study type	5.14	5.66	2027882
3	Study type	3.47	33.14	622247

CHAPTER 5

DISCUSSION

5.1. GROWTH AND SECONDARY METABOLITE STUDIES WITH A *PHOMA* SPECIES USING GRAIN AND SEED EXTRACTS.

This study was designed to be preliminary to work with solid substrates, and utilised an organism producing a metabolite of pharmaceutical relevance. The use of grain and seed extract media was valuable since it gave an insight into the types of materials that could be usefully employed as solid substrates. In fact these extracts represented simplified versions of solid substrates, being essentially homogeneous in nature, and allowing direct quantitative measurement of mycelial growth responses. This is not currently possible with SSF, although a number of indirect methods have been described.

5.1.1. Effects of substrate type, a_w and temperature on growth.

In this study, growth of the *Phoma* species was shown to respond strongly to temperature variation in the range 15-25°C in comparison to the response to a_w in the range 0.998-0.96. However, growth was significantly reduced at 0.96, except for MEA at 25°C. Maximum growth rate was recorded at 25°C which has previously been reported as the optimum growth temperature for this organism (Baxter, 1997), and the consistently higher growth rates at this temperature at all a_w levels reflect the findings of Ayerst (1969), that the greatest 'tolerance' to lowered a_w occurs at the optimum growth temperature.

On the nutritionally rich MEA a dense assimilative type growth form was evident, whilst on the more nutritionally depleted grain extract media an effuse explorative growth form was noted, characterised by rapid linear expansion as defined by Rayner (1994) (see Plate 4.1.). This may represent a physiological adaptation effected by the organism in response to the nutritional status of the environment (Cooke and Whipps 1993). Although linear growth rate was greatest for the grain-derived media, biomass production was probably greatest on MEA.

5.1.2. Effects of substrate type, a_w and temperature on temporal squalestatin production.

Most notably, this study has indicated that squalestatin S1 production responded to two environmental stress effects. Firstly, the imposition of water availability stress (in terms of lowered a_w) and secondly nutritional stress (as imposed by nutritionally depleted grain extract media when compared to MEA). Although ANOVA revealed a significant effect of temperature on S1 production overall, LSD analysis revealed no discernable trends and it was concluded that temperature within the range 15-25°C did not significantly influence S1 production. This is in marked contrast to the effect of temperature on growth. Baxter *et al.* (1998), working with the same organism grown on modified MEA only, reported a temperature optimum of 20°C for S1 production at 0.998, 0.995 and 0.99 a_w levels, and no significant temperature effect at 0.98 a_w .

The effects of the use of nutritionally depleted media on S1 production are probably the most straightforward to interpret, since these are least likely to impose a

significant catabolite repression effect, and may therefore be expected to produce metabolite earlier and at higher levels than those observed for MEA. Differences between the two starchy grains (wheat and oats) probably reflect the balance of nutrients and their accessibility during extraction - oats in particular is noted as having a tough outer 'husk' (Hajjar *et al.* 1985). The results for oil seed rape may reflect the high lipid content, which is presumably unsuitable for this particular organism, although it is rapidly utilised by spoilage fungi (Magan *et al.*, 1993). It is also possible that crude seed extracts contain uronic acid oligosaccharides which have been implicated in increased metabolite production (penicillin G) by *Penicillium chrysogenum* strains, although interactions with water availability were not examined (Asilonu, 1999). It has been suggested that modulation of gene function could occur following binding of such oligosaccharides to the surface of the hyphal cells. This could lead to increased cell activity on entry into the cytoplasm, including increased biomass production and biosynthesis of secondary metabolites, especially in liquid culture systems (Asilonu, 1999).

The production of higher titres of S1 at lowered a_w levels is in marked contrast to the growth data where the maximum growth rate was recorded in the range 0.998 - 0.995 a_w , and appears to be an example of metabolite production responding specifically to water stress. It is also possible that the glycerol used to modify low a_w treatments had a direct stimulatory effect on S1 production; Northolt and Bullerman (1982) found that glycerol had a stimulatory effect on the mycotoxin aflatoxin B1 (which, like S1 is a polyketide). Further, Connors *et al.* (1995) linked increased glycerol utilisation with

increased zaragozic acid production in submerged fermentations of *Leptodontidium elatius*. However, in the present study the same pattern of results was obtained for all media types, including MEA where the influence of glycerol as a carbon source would be expected to be less marked, since it is unlikely to be preferentially utilised in the presence of simple sugars. In addition, Baxter (1997), working with the *Phoma* sp. reported a similar pattern of results when growing the organism on maize grits, over a range of a_w levels without the use of glycerol.

The effect of water availability stress on S1 production is particularly interesting. A key point here is that the manipulation of a_w in a range only slightly inhibitory to growth has resulted in a significant enhancement of metabolite production. In many previous studies, large a_w changes have been imposed so that growth and, not surprisingly, secondary metabolite production were both strongly inhibited. However, there are some studies that have reported enhanced production at a_w levels lower than those optimal for growth. For example, Gervais *et al.* (1998) studied the production of 2-heptanone (a volatile metabolite) by *Trichoderma viride* on a yeast starch agar medium and reported optimal production of the metabolite at 0.97 - 0.96 a_w , with optimal growth at 0.995 a_w . Similarly, Baxter *et al.* (1998), working with the *Phoma* sp. grown on glycerol-amended MEA reported optimum a_w for growth and metabolite production at 0.995 and 0.995 - 0.980 respectively.

Lowering of a_w can be seen as a stress effect with direct relevance to the natural environment, in terms of the onset of drought conditions. Drought represents a major

difficulty for soil-borne organisms such as the fungus used in this study, so it is perhaps not surprising to find a regulation mechanism which appears to respond to this stimulus.

Metabolite responses to environmental stresses presumably heighten the competitive advantage of the producer in the face of deteriorating environmental conditions, assuming that the metabolite(s) so formed have a relevant extrinsic biological function. In fact, catabolite repression itself can be viewed as a specific mechanism that has evolved to respond to nutrient stress, i.e. carbon depletion, and triggers metabolite production at a time when the organism needs to gain a competitive advantage.

In the case of nutrient depletion, high levels of secondary metabolite production were accompanied by a specific, diffuse “foraging” growth form designed to “explore” as much of the environment as possible, with minimum biomass production. This is accomplished by the restriction of branching, and maximum elongation of existing hyphae. The adoption of this growth form, with the concomitant increase in metabolite production, represents a remarkably sophisticated response by the fungus.

The recognition of these effects has practical implications for the production of fungal extracts for screening programmes. At present little emphasis is placed on the optimisation of environmental conditions in terms of precise a_w control or the imposition of nutritional stress. Both of these factors have been ignored in the conventional use of established commercial media (Penn, 1994). Tribe (1987) has also

previously questioned the wisdom of the use of the mycologists' standard range of rich media. This study has suggested that some systems may benefit from the manipulation of environmental parameters so that the organisms are stimulated to effect the secondary responses that are normally tightly regulated under 'ideal' growth conditions.

5.2. INITIAL WORK WITH SOLID AGROFOOD SUBSTRATES.

5.2.1. Solid substrates used in the study.

Following on from work with a *Phoma* species using homogeneous extracts of agricultural materials, this phase of the project involved initial investigations of intact solid substrates, to enable a choice to be made of a suitable material for further work. Agricultural materials were an obvious choice for this work. They have been used extensively as solid substrates for fungal fermentations. For example, Magan and Lacey (1984 a,b,c, 1985), have made numerous studies on mycotoxin production by various fungi on agricultural materials, and Hesseltine (1972) has reported the use of agricultural materials for production of aflatoxin. In fact, it would be very difficult to make a sensible choice from any other class of materials. There has been some limited use of synthetic and mineral materials in SSF (Kerem and Hadar, 1993; Durand *et al.*, 1996), and this project does utilise a mineral medium as a second "phase" in some experiments. Another choice may have been waste materials made available by the food processing industries. Many materials such as beet pulp, sugar cane bagasse and

potato residue find application in SSF (Nigran and Singh, 1996; Smits *et al.*, 1998).

Of course, these may also be considered as agricultural materials, but they are not derived directly from the agricultural industry since they are, by definition, processed substances. Wastes were not considered suitable materials for the present type of work. This was due to the following main reasons:

1. They can be expected to be (in part at least) nutritionally “spent” and could present nutritional deficiencies significant for secondary metabolite production.
2. They are likely to be highly variable between batches, and possibly not always readily available.
3. They are likely to be contaminated with foreign materials (e.g. machine oils) and undesirable micro-organisms.
4. They would be unacceptable to the pharmaceutical industry as feed-stock for pharmaceutical production or development. This is due in part to the very stringent requirements of Good Manufacturing Practice (GMP) which the pharmaceutical industry is subject to. GMP requirements extend to the raw materials used in drug manufacture, and are far more exacting than the standards required in food production (Dr. S. Martin, TerraGen Discovery Ltd., pers. com.).

On the basis of nutritional status, all the materials chosen seemed reasonable candidates as solid substrates. All represent human food in one form or another, and all can, and will, support fungal growth if improperly stored. The materials chosen fell into a number of distinct categories: starchy grains, oily seeds and processed materials derived from wheat (a starchy grain). Wheat and wheat derived products figured strongly in the choice of substrates since this material (in the form of an extract) had supported high squalenol production in the *Phoma* species study earlier in this project. The nutrient balance of the starchy materials (raw and processed) varied by only a limited degree, with some notable variations (e.g. rice had significantly lower levels of protein and fat contents at 7.7 and 0.6% respectively, and oats and flaked millet had relatively high fat contents at approx. 5%). Of course the nutrient analysis given for these substrates was limited in as much as no information was available for specific minerals, vitamins and trace elements, which could be significant for both fungal growth and secondary metabolism.

The particle size range for this collection of materials varied from 10 x 9 mm. for maize kernels to approx. 2 mm. for couscous "grains". Previous studies have reported a number of particle sizes near or within this range as "ideal" for SSF. Pandey (1992) reported up to 1.4 mm. (cassava starch) as optimal for enzyme production. Hesseltine (1972) reported whole rice or wheat (approx. 6 – 7 mm. length) optimal for aflatoxin production by *A. parasiticus*. Barrios-Gonzalez *et al.* (1993) reported optimal gibberellic acid production on wheat bran of 3 – 4 mm. In practical terms, there is likely to be a specific optimal particle size range for both growth and metabolite

production with both of these parameters restricted outside of these limits. The upper limit is likely to be governed by considerations of surface to volume ratio i.e. the surface area available for fungal growth, and the diffusion of nutrients. The lower limit is most likely to be due to the “packing structure” of the substrate and the nature of the void or pore space produced. As the particle size becomes smaller so does the pore size, until a cut-off point is reached where the mass as a whole can quickly become oxygen limited during microbial growth. This relates to the amount of oxygen that can be held in the mass and the efficiency of oxygen transfer. Substrates of very small particle size may also present practical handling difficulties due to a tendency for “caking” and “clogging” during use. Packing behaviour, and in particular packing density will be affected by particle shape, since flat, flake-like particles are likely to pack with a far greater density than more regular shaped particles. In fact, this effect was noted with wheat flakes. Flatter particles will therefore tend to compact, effectively excluding pore space and causing oxygen limitation (Barrios-Gonzalez *et al.*, 1993).

The inclusion of some processed materials was considered important in this study. In general, it is likely that most substrates in SSF benefit from some form of prior processing such as steaming, autoclaving, pearling or cracking and chopping. These processes have a number of important effects on surface characteristics:

1. Protective outer layers (pericarp, husk) are removed (e.g pearling of wheat grain)

2. "Cooking" processes break down the structure to a degree which renders nutrients more available.
3. By breaking substrates into uniform sized particles degradation efficiency is increased and pore structure developed.
4. Broken and abraded surfaces are produced. This appears to be important for microbial attachment and penetration (Knapp and Howell, 1980).

For the current purposes, processed materials were defined as those that have undergone specific heating and/or mechanical operations other than de-husking, pearling or cleaning. Processed materials were therefore flaked wheat, flaked millet, bulgar wheat, couscous, "shreddies", "weetabix" and "shredded wheat". Of these materials, all except "shreddies" and "weetabix" responded well to wetting and autoclaving. These two could be described as over-processed for the current purposes, since they softened too readily – a characteristic presumably designed to produce an acceptable breakfast cereal product. Products such as bulgar wheat and couscous had a far more resilient structure which was able to withstand repeated autoclaving without loss of integrity. Again, this probably reflects the intended use of these materials which are cooked and eaten with meat or vegetables and designed to retain a particular "mouthfeel" similar to rice. This resilience is a characteristic of durum wheat, from which these products are usually made. (Dick & Matsuo, 1988).

From considerations such as those discussed in this Section, it is possible to put forward a list of criteria for an “ideal” substrate for SSF. Such a list would include the following:

1. Adequate nutrient status. Not necessarily nutrient rich, as pointed out by Tribe (1987). Optimal nutrient status may be process specific.
2. Good “granulometry”: uniform particle size, structural integrity (important in preparation, during fermentation and in subsequent processing), good void structure.
3. Prior-processed (see discussion on pre-processing above).
4. Available throughout the year and of consistent quality.
5. Economical, probably least important for a high added value product like a pharmaceutical.

5.2.2. Moisture sorption isotherms.

Moisture sorption isotherms predict little about the suitability of a substrate for SSF. However, they are essential tools, allowing a_w to be set accurately in subsequent work. For a number of the substrates used in this project, detailed moisture sorption isotherms were constructed for the first time. The isotherm results were surprisingly uniform across the materials tested. They fell into two broad groups: the raw materials

and the processed materials. The raw materials demonstrated 0.90 a_w values in the range 15 – 20% moisture, 0.95 a_w in the range 20 – 25% and saturation at around 40% moisture. The similarity of these results within the starchy type materials is perhaps not surprising since the compositions of these as a group were generally quite similar. It is more surprising that dissimilar materials such as oil seed rape should fall into this group. The second identifiable group, the processed materials, all demonstrated lower a_w levels for a given moisture content compared to the raw materials (e.g. typically 0.95 a_w at approx. 30% moisture). In general, this may reflect the physical and chemical changes brought about by processing. Mechanical processes designed to produce uniform particles will produce a much larger surface area capable of physical interaction with water. Cooking processes may alter chemical constituents so that they interact differently with water (e.g. cooking of bulgar wheat to gelatinise the starch may have this effect).

The isotherms obtained for maize, wheat, rice and oil seed rape were in broad agreement with previously published isotherms for these substrates (Multon, 1988). There was less agreement for soya (in the same publication) which showed generally a higher a_w for a given moisture content than obtained in this work. Some variation is to be expected since cultivars were presumably not the same and isotherm preparation temperatures varied in some cases.

For the most part, the preparation of moisture sorption isotherms was straightforward, although time-consuming due to the lengthy period required for the a_w meter to reach

equilibrium. However, the construction of an isotherm for perlite proved impossible since any addition of water to this substrate gave a reading indicating saturation (1.00 a_w). At first the meter was suspected of being faulty, until it was realised that perlite cannot be expected to behave in the same way as the other materials. Perlite is an inert mineral lacking all of the (biological) properties that would cause water in contact with it to partition into "free" and "bound" states. For water associated with perlite there is no "bound" phase because the strong physical and chemical interactions do not occur. This is presumably why perlite can be used in horticulture as a water donater.

5.3. SMALL SCALE GROWTH AND METABOLITE STUDIES.

5.3.1. Effects of substrate type and a_w on growth.

This phase of the project involved investigation of growth and secondary metabolite production by the four "tropical" fungi on a range of substrates. Although certain features of growth were noted, biomass production was not considered to be of major importance for the purposes of this study. This was mainly because earlier work with the *Phoma* species had indicated that there was not necessarily any clear link between extent or quality of biomass and secondary metabolism.

Overall, growth responses were relatively uniform between substrate types for a given fungal species. This is perhaps not particularly surprising since the majority of the substrates were reasonably similar starchy materials, although parameters such as

particle size and type of prior processing differed widely within this group. It is more surprising that materials with less similar compositions (e.g oil seed rape and soya) produced broadly similar growth responses (oil seed rape produced slightly less growth for *D. dematioidea* and *S. palmicola*). These results probably reflect the general adaptability and opportunistic tendencies of most fungi in colonising “whatever is available”. Further, it should be borne in mind that the essentially qualitative type parameters used to rate growth in the present study were crude and not particularly discriminating. They represented a compromise to circumvent the difficulties of measuring biomass in SSF. In fact, there have been a number of methods put forward to measure biomass in solid cultures (e.g. Zhu *et al.*, 1994, Baxter, 1996) but this was not considered a primary concern in the present work. Further, the step-down in temperature (25°C to 18°C) at the mid-point of incubation could be expected to have an inhibitory effect on growth. This was designed as a “temperature stress” effect to attempt to stimulate metabolite production. In effect, growth was deliberately compromised in these studies for this purpose.

Although growth responses to lowered a_w differed markedly between fungal spp., responses across the range of solid substrates used for a given fungus were reasonably uniform. Growth responses to lowered a_w were most notable in *D. dematioidea* where a change in sporulation pattern occurred, and in *S. palmicola*, where growth of spore bearing structures was severely inhibited. It should be borne in mind that the manipulation of a_w level in this study was intended to be a relatively subtle effect rather than a severe one. The generally slight changes in growth responses, for most

organisms, across the a_w range employed indicate that this has been accomplished reasonably well.

5.3.2. Two-phase studies.

The early studies in this phase of work, including medium A, wheat, oats and OSR, all involved the use of a second medium phase (perlite in most cases) and nutritional supplements added to the media components. Nutrient supplements were added to supply components that may have been missing from the substrates themselves, and the second inert substrate was included to present a nutritional “phase change” to the growing organisms that, it was believed, could stimulate metabolite responses. However, it was not known how significant these additions were, and so a series of experiments was carried out where these elements were added separately to a common substrate (wheat flakes). The results did not indicate a significant effect of using either nutrient additions or an inert second phase on growth or metabolite production, and these additions were not used in subsequent work. In fact, the absence of additional nutrient elements may be more advantageous for metabolite production, by providing imbalanced conditions, as suggested by Maplestone (1992). Of course, “imbalanced” conditions may be interpreted as “stressed” conditions, which is a central theme in the current work.

The use of an inert second phase represents an interesting idea, and in some experiments there did appear to be some accumulation of pigment at the phase boundaries (perhaps made clearer due to the white colour of the perlite). It seems

likely however that diffusion of nutrients during autoclaving and incubation would eliminate any significant nutrient boundaries between the phases.

5.3.3. Effects of substrate type on metabolite production.

In contrast to the growth trends observed, the results for metabolite production were strikingly different between media types for all organisms. For example, for *C. cassiicola*, 5 peaks were produced by soya and oil seed rape and no peaks were recorded for maize. *D. dematiodea* produced 9 peaks for medium A and 4 for oats. *S. palmicola* produced 6 peaks eluting before 8 min for medium A and none for rice. *E. nigrum* produced 7 peaks eluting before 9 min for medium A and only one for wheat flakes. The differences are even more extreme if wheat flakes is compared with bulgar wheat or couscous in which all peaks obtained are shown. It could be argued that some of the variation observed was due to changes in the states of the organism between studies (e.g. genetic drift). However, this possibility was minimised by the use of single master cultures which were sub-cultured only once for each experiment, and discarded after six months. They were then replaced from cryo-preserved stocks.

The effect of using different substrate types on secondary metabolism in solid state fermentation (SSF) has been investigated by a number of workers. Generally, the variable metabolite responses noted in the current work have been mirrored by previous work. For example, O'Neil *et al.* (1993) studied the production of zearalenone and two other mycotoxins by *Fusarium* spp. on maize, rice, wheat and barley and found significantly greater production on maize and rice. Presumably they

used whole grains, since they attributed these differences to the seed coat on wheat and barley which they believed slowed fungal development. Hajjar *et al.* (1985) grew various strains of *Aspergillus nidulans* on rice, oats and a defined liquid medium, and assayed for sterigmatocystin production. All strains produced the toxin on solid substrates with yields ranging from 4.6 - 32.6 $\mu\text{g g}^{-1}$ for rice and 9.0 - 79.3 $\mu\text{g g}^{-1}$ for oats, but only one strain produced the toxin in the defined medium. Barnes *et al.* (1993), found that when comparing oats, rice and "shredded wheat" as media for *Aspergillus* and *Chaetomium* spp, maximum production of sterigmatocystin was produced by *A. versicolor* on "shredded wheat".

Chulze *et al.* (1990) studied the growth and production of aflatoxin by *Aspergillus parasiticus* on sunflower seeds and reported enhanced production of the toxin on this oily substrate compared to starchy seeds. They found that the saponified fraction of fatty acids in sunflower oil, and the unsaturated fatty acids associated with it, had a strong influence on mycotoxin production, yielding higher levels of toxin than a preparation of defatted acids. However, in the production of two alternariol mycotoxins by strains of *Alternaria alternata*, Chulze *et al.* (1994) found that a starchy substrate (rice) was superior to sunflower seeds. It therefore seems that the behaviour of fungi on solid substrates with respect to secondary metabolite production is very specific to the particular producing organism and metabolite involved. Variation is probably due to a number of factors, including the ability of the producing organism to utilise the medium, in a far more subtle way than is manifested by any changes in growth patterns, and the availability of trace elements and perhaps specific precursors

required for metabolite production. Levels of specific repressor or other regulatory molecules will also exert a significant effect.

5.3.4. Effects of a_w on metabolite production.

Manipulation of a_w produced reasonably uniform responses in metabolite levels for a given fungal sp. For *C. cassiicola* and *D. dematioidea*, a_w had the least effect with metabolite levels generally drifting down with lowered a_w . For *S. palmicola*, dramatic reductions in metabolite levels were observed with decreasing a_w . For *E. nigrum*, a significant number of metabolites were stimulated by decreased a_w . This is particularly clear in the profile for medium A in which virtually all metabolites are produced at significantly higher levels for a_w values below 0.998. Indeed, some metabolites only appear at the lower levels, and represent unique metabolites obtained in the current work. Optimum metabolite production in this profile is shown to occur at 0.98 a_w . Taking all profiles into account, optimum key metabolite production for this fungus probably occurred in the range 0.99-0.98 a_w .

The different responses of *E. nigrum* and *S. palmicola* to a_w manipulation are particularly interesting. *E. nigrum* is an extremely cosmopolitan phyllosphere dweller which can be expected to encounter water stress, in the form of drought, in its natural environment. According to a classification proposed by Griffin (1981), *E. nigrum* was described as sensitive to changes in a_w , and Magan and Lacey (1984a), described the lower limit for growth of this organism as occurring at 0.89 a_w (25°C). Subtle manipulation of a_w , producing mild water stress resulted in a strong response in terms

of increased metabolite levels. However, *S. palmicola* was isolated from an essentially aquatic habitat (water-logged palm fruit from rain forest swamp), and both growth and secondary metabolite production were strongly inhibited in the same range of a_w levels. This isolate of *S. palmicola* was probably not adapted to respond to lowered water availability conditions, so that the stress imposed was both severe and inappropriate, in consideration of its natural "lifestyle". It is noteworthy that this organism also showed growth limitation at lowered a_w . This leads to a general suggestion that the imposition of stress effects, to illicit metabolite responses, needs to take into account the natural environment of the organisms, i.e. the stress effects need to be *relevant*. This type of argument is in keeping with the "chemical ecology" approach to natural product discovery as discussed by Caporale (1994), and further underlines the value of the manipulation of relevant environmental parameters in natural product screening programs.

There have been many studies carried out on secondary metabolite production at varying a_w levels, mostly concerned with mycotoxins and utilising both solid substrates and agar media (see Section 1.4.). The vast majority have reported a reasonably straightforward decline in production with lowered a_w , but typically they have used wide a_w ranges which may be expected to have severe effects. There are, however, some reports of secondary metabolite stimulation with lowered a_w , a notable example being Montani *et al.* (1988), working with *Aspergillus parasiticus* growing on viable corn kernels over the a_w range 0.97 - 0.87, who reported maximal aflatoxin accumulation at the relatively low a_w level of 0.90. Also, Cuero *et al.* (1987), working

with similar organisms, reported high levels of ochratoxin A production by *Penicillium viridicatum* over the a_w range 0.98 - 0.90. Again, the wide and presumably severe a_w ranges employed in these studies is noteworthy, and it is interesting to speculate on the possible outcomes, if more “subtle” a_w ranges had been employed.

In this study, metabolite production appeared to be strongly influenced by nutrient status, and in the case of *S. palmicola* and *E. nigrum*, also by manipulation of a_w . This was in contrast to the effects of these parameters on growth, except perhaps for growth of *S. palmicola* at lowered a_w , where a clear effect was noted. This indicates that secondary metabolism is a very finely balanced process, responding to subtle variations in environmental parameters which do not illicit a parallel response in growth. This is in keeping with the work conducted in the *Phoma* species, where growth and secondary metabolite production followed entirely different patterns in response to manipulation of nutrients and a_w .

5.3.5. Choice of a model system.

The choice of an organism to use in further work was reasonably straightforward. Of the four organisms tested, *E. nigrum* produced metabolite profiles most faithful to the profiles originally obtained by TerraGen. The most encouraging range of target metabolites was also obtained with this organism, as well as the most positive response to lowered a_w conditions. This latter response was considered important in consideration of SSF scale-up, where the ability to manipulate substrate a_w could be particularly important. Further, certain strains of *E. nigrum* are known to produce antimicrobial compounds of possible pharmaceutical interest, one example being

flavipin. Antimicrobials such as this are probably important in the use of this organism as a biocontrol agent (Pascual *et al.*, 1996). Strong pigment production in this organism was also a useful “marker” in following the progress of fermentations. Zhou *et al.* (1996) have in fact previously suggested the use of pigment production as a preliminary screening tool for natural product discovery.

The choice of a suitable substrate to use in subsequent work was less straightforward. On the basis of target metabolite range and levels, the most obvious choice would have been whole wheat grain. However, bulgar wheat was ultimately chosen as it was considered that it best fitted the criteria for “ideal” substrates detailed in Section 5.2.1. In particular, it demonstrated the following attributes:

1. Good “granulometry”; uniform particle size of approx. 3 mm, free flowing over a wide range of a_w levels.
2. Good structural integrity, able to withstand repeated autoclaving cycles.
3. Pre-processed; semi-cooked with outer layers removed, and abraded surface characteristics.
4. Freely available at consistent quality.

Wheat, in fact generally appears to be a good substrate for metabolite production in fungal SSF. For example, Kobbe *et al.* (1977) found it to be the best of a range of starchy substrates for production of malformin C by *Aspergillus niger*, and Ghosh *et al.* (1978) found it superior to a similar range of starchy grains for production of simatoxin by *Penicillium islandicum*. Further, wheat extract produced the highest titres of squalestatin by the *Phoma* species used earlier in this project. It was felt that

bulgar wheat, as a particular type of processed wheat product, would offer most potential as a solid substrate for further work.

5.4. INVESTIGATION OF TEMPORAL DEVELOPMENT OF METABOLITES.

5.4.1. Inoculation technique.

The use of pre-inoculated bulgar wheat as the inoculum in this study represented an improvement over the previous methods. In particular, pre-grown bulgar wheat facilitated rapid colonisation of the substrate after inoculation. The main advantage of this method seemed to be that the organism was physiologically pre-adapted to the substrate so that no significant lag phase was observed. In addition, this method did not involve addition of a liquid medium which would cause alteration of substrate a_w . The distinctive colour of pre-inoculated material was also an important advantage in helping to assess the uniformity of distribution of the inoculum particles in the substrate mass. This was expected to become an important consideration as the scale of the fermentation processes increased.

5.4.2. Changes in culture media a_w .

The increase in a_w levels of the media during the course of the fermentations was not perhaps a surprising result in itself and presumably resulted from an accumulation of metabolic water as the fungus developed. Dorta and Arcas (1998), working on SSF of

Metarhizium anisopliae on a rice based medium have reported a similar finding, with moisture content rising from 47% w/w to 60% w/w over a 10 day period. It should be noted that during the course of a fungal SSF process, the physical and chemical make-up of the substrate will change progressively as the material is degraded and biomass accumulates. Oriol *et al.* (1988), working on SSF of *Aspergillus niger*, reported the a_w of the substrate rising from 0.94 to 1.00 in 22 hr. They attributed this mainly to the a_w of the developing fungal biomass, and claimed that the substrate phase itself probably became drier as the fermentation progressed. However, the obvious accumulation of water which occurred in the present work would seem to refute this claim. The changes in measured a_w are not surprising when viewed in this light, but lead to two important practical considerations:

1. a_w stress effects imposed at the start of the fermentation are likely to decline with time. However, from Figure 4.19. it is probable that treatments starting at approx. 0.96 and 0.98 will remain significantly stressed for the duration of a two week fermentation. Interestingly, the fungus must initially overcome these stress effects to produce the biomass necessary to lift a_w . Fungi that are adapted to grow in low a_w conditions presumably respond by production of compatible solutes, while others will fail to grow.
2. Water accumulation may be sufficient to adversely affect the outcome of the fermentation process. This could occur if water began to fill the void spaces and

caused O₂ limitation (Moo-Young *et al.*, 1983). Excess water could also encourage bacterial contamination in systems that are not maintained strictly aseptic.

5.4.3. Bacterial contamination.

The main difficulty experienced in this study was bacterial contamination. This affected all of the wettest statically incubated treatments (0.998 a_w), and caused the abandonment of this part of the experiment. Since all treatments had been autoclaved as a single batch, a sterilisation problem affecting only these treatments was considered unlikely. Similarly, a contamination problem occurring during inoculation was unlikely to have affected only these treatments. It was considered probable that the whole experiment had been subject to bacterial contamination, due to a small residual population persisting in the autoclaved material, but that this had only manifested itself at the highest a_w level, bacteria being essentially aquatic, and presumably inhibited in dryer conditions. The 0.998 a_w shaken treatment may have avoided this type of obvious contamination due to a lower accumulation of water. These treatments were incubated in an unlidded reciprocating shaker so were subject to greater evaporative losses than the static treatments. At this stage it was unclear whether this was likely to be a persistent problem, or if it was peculiar to this study.

5.4.4. Temporal development of metabolites.

Above all, this study underlined the varying temporal development of those metabolites observed. Metabolite 1 accumulated up until day 24, while metabolites 2

and 3 reached peak levels by day 7. Levels of metabolites 2 and 3 then remained constant for the duration of the study, suggesting that they were relatively stable, while the additional metabolite eluting at 8 min declined beyond day 10, suggesting that it was less stable. Temporal development and stability were therefore shown to be metabolite specific. These points indicate the importance of conducting temporal studies in determining the optimum duration of a fermentation process for a given product. For the present system, these results indicate that for metabolites 2 and 3 the fermentation need run no longer than 7 days, but for metabolite 1 a fermentation of 20 days duration is required for optimum product accumulation.

The increased production of metabolite 1 with lowered a_w is in keeping with previous studies in this project, as is the appearance of further metabolites at 0.97 a_w . These results underline the potential of the use of low a_w conditions for the production of enhanced levels of metabolites, at least in some systems. The results obtained also suggest that there is little value in stepping down the temperature from 25°C to 18°C at the mid-point of incubation, as had been practised in previous studies in this project.

The strikingly different response of the shaken treatment was particularly interesting. The development of little obvious hyphal growth accompanied by a marked and early development of pigment was very similar to the observations made by Hesseltine (1972), working on aflatoxin production by *A. parasiticus* on shaken cultures of rice and wheat. Hesseltine (1972) in fact reported a very clear pigment progression to a dark brown colour, most noticeable in rice. The high metabolite levels he obtained in

his system were also reflected in the levels obtained here. The different metabolite profiles may have resulted from hyphal shear stress, or by the improved aeration characteristics of these treatments. It is significant that the organism appeared to tolerate intermittent shaking of this type, in as much as metabolite responses were not inhibited; Barrios-Gonzalez (1993) has previously noted that some fungal species cannot tolerate any form of agitation in SSF. The apparent ability of the organism to tolerate shaking was considered important with a view to subsequent method development and the design of scale-up systems. In fact, the markedly different metabolite profile obtained under shaken conditions suggested the possible use of this technique for the production of enhanced levels of specific metabolites.

5.5 INVESTIGATION OF THE EFFECTS OF AERATION AND AGITATION AT SMALL SCALE.

5.5.1. Metabolite profiles.

This study, conducted at small (jar) scale was carried out to investigate the effects of forced aeration and intermittent agitation on metabolite production. This was considered important with a view to scale-up, where either or both of these operations could be important in process control. Barrios-Gonzalez and Tomasini (1996) have previously reported increased aflatoxin production under conditions of forced aeration, and Ellis *et al.* (1994) have described a strong response in aflatoxin production to O₂ level. However, in this study no similar enhancement of major target metabolites (metabolites 1, 2 and 3) was noted. In fact, for metabolites 1 and 3

significantly higher levels were produced for the statically incubated treatment. This may have been a “non specific” effect resulting from the local drying experienced by the aerated treatment, which prevented growth adjacent to the point of air entry. This was in spite of the humidification of the input air, and suggested the need for damper air in any subsequent work requiring aeration. It was concluded that statically incubated treatments *at this scale* were not O₂ limited, at least for the metabolites of interest in the current study, and there was no clear advantage in the provision of excess O₂. However, forced aeration appeared to have the potentially useful side effect of causing general drying which, if controlled properly, could possibly be utilised in preventing excess accumulation of metabolic water.

Previous work in this project had indicated a strikingly different pattern of metabolite development under agitated conditions (Section 4.4.). It was thought that this may have arisen due to increased aeration, or as a result of shear stress. However, in the present study no dramatic changes in patterns of metabolite development were noted for the agitated treatment. The effect noted here was a significant decline in metabolite 1, similar levels for metabolite 3, and an absence of the other two metabolites that appeared in the aerated and static treatments. Obvious differences between the shaken treatment in this study and the previous shaken experiment (Section 4.4.) were the mode of shaking and the initial a_w of the substrate. In the previous experiment shaking had been continuous and relatively gentle, while in this experiment the shaking was intermittent and severe, possibly causing far greater shear damage. The initial a_w of the substrate in this experiment was 0.990 compared to 0.998 in the previous

experiment. It is not clear what effects this could have on the response to shaking. Perhaps the combined stresses of low a_w and high mechanical shear were sufficient to bring about a general inhibition of (some) metabolite levels. As has been noted previously, these effects, whilst having a significant impact on metabolite production, did not appear to be sufficient to have a similar effect on growth.

It was concluded that intermittent agitation was neither advantageous nor desirable for production of the metabolites in question, but could perhaps in principle be tolerated if required in the development of scaled-up systems.

5.6. FIRST SOLID SUBSTRATE SCALE-UP STUDY OF *E. NIGRUM* WITH BULGAR WHEAT.

5.6.1. General observations.

This study represented scale-up of one order of magnitude compared to previous experiments. Scale-up was kept as simple as possible and no modifications to conditions or systems were imposed at this stage. In particular, no effort to retain “geometric similarity” was attempted, which has previously been suggested as a simple method for SSF scale-up (Akao and Okamoto, 1983). The approach adopted here allowed a direct comparison between this study and previous small scale studies.

5.6.2. Water accumulation.

Inoculation with pre-grown bulgar wheat was again successful and demonstrated the advantages previously discussed. However, the steady build up of water which followed inoculation soon became a significant problem. Clearly, the problem was greatest for the treatments that started wettest, as these quickly became water-logged, but water accumulation affected all treatments and underlined the need for some form of water content control in these fermentations. Shaking of bottles (in the case of the shaken treatments) had the effect of re-distributing the moisture i.e. it was cleared from the vessel walls, but it was still retained in the substrate bulk to no real advantage. It was apparent that this first level of scale-up had exacerbated the problem of water accumulation, first noted in the temporal study (Section 4.4.), perhaps partly because of the greater substrate bulk and the proportionately smaller surface available for evaporation compared to small scale (as observed by Lonsane *et al.*, 1992). It was therefore obvious that any further attempts to scale-up this system would need to address the problem of water accumulation.

5.6.3. Heat build-up.

Temperature control was unsatisfactory in this study. Bottle temperatures (as simply measured by glass wall temperature) indicated a temperature differential of approx. +5°C compared to incubator temperature. This was almost certainly an underestimate of overall temperature increases, as internal temperatures could be expected to be far in excess of this. This is certainly not a surprising result and is well documented in the literature (e.g. Lonsane *et al.*, 1992; Ghildyal *et al.*, 1993). However, such temperature

differentials have a number of effects on growth, productivity and moisture distribution and all lead to an increasingly heterogeneous system which is not under adequate control. Bottle shaking probably had the effect of mixing warmer and cooler zones and so re-distributing the heat to some extent. This however had no significant overall cooling effect, as shaken bottles were equally as warm as static bottles for the entire duration of the study. Similarly to the problem with water accumulation, heat build-up needed to be addressed in consideration of any further scale-up work.

5.6.4. Bacterial contamination.

Bacterial contamination was a significant problem in this study. Again, the worst affected treatments were those with the highest initial a_w (e.g. the 0.998 a_w shaken treatment abandoned by day 9). It had been concluded previously (Section 5.4.3.) that the bacterial contamination probably persisted in the substrate at low level after sterilisation. This was borne out by the results of this study. The bacterial contamination appeared to be a *Bacillus* species, some of which are capable of producing heat resistant spores that could potentially survive lengthy autoclave cycles when "protected" in a solid matrix. Further, the growth rate of the organism (as evidenced by overnight growth on NA plates) was quite remarkable, and it is conceivable that just a few surviving spores could develop into gross contamination in a few hours, under the right conditions. Bacterial spread would be facilitated by shaking, and it is noteworthy that the shaken treatments exhibited contamination earliest in this study. The critical factor in the development of the contamination seemed to be the water content of the substrate. Growth of the bacterium was

triggered after a number of days of incubation, corresponding to the attainment of a critical water level in the substrate. Such a situation would explain why bacterial contamination affected the high a_w treatments while the low a_w treatments appeared unaffected, and would also explain why sterilised, uninoculated material appeared to “keep” indefinitely. Bacterial contamination represented a significant problem at this stage since it appeared that batches of substrate as small as 400 cm³ could not be successfully sterilised by two autoclave cycles. This was particularly disappointing and threatened the viability of the whole system, particularly with a view to scale-up. The most obvious next step appeared to be to extend the autoclaving cycle time for subsequent substrate preparation.

5.6.5. Growth and metabolite profiles.

Not all aspects of this study were negative. Treatments that were completed successfully demonstrated uniform fungal growth and good pigment development throughout the whole substrate mass. This uniformity of growth suggested that there was no significant gas transfer limitation at this scale, even for the statically incubated treatments. Further, the pattern of metabolite development was similar to that seen previously in small scale experiments. The levels of metabolites were very encouraging, particularly for metabolite 1, although variation between replicates was quite large, as shown by the error bars in the relevant figures. Comparison between metabolite levels in static and shaken treatments indicated that shaking did not have a significant effect on the outcome of the fermentations. This was at variance with previous results obtained with shaken incubation. In the first study involving shaking

(temporal study, Section 4.4.), agitation stimulated the production of a markedly different metabolite profile. In the second study (Section 4.5.), agitation produced a general inhibition of metabolite levels. It is tempting to suggest from this that shaking the cultures lead to a highly delicate situation where the degree of stress imposed resulted in a number of outcomes, depending on severity. It was certainly clear that an already finely balanced system, in terms of metabolite responses, was made even more unstable by the addition of agitation. However, these results continued to demonstrate that the present system would tolerate a certain degree of agitation.

5.7. FIRST LABORATORY SCALE FERMENTER STUDY.

5.7.1. Method development.

This study was designed primarily around the need to introduce a system for water and heat removal during fermentation. A number of options were considered to accomplish this:

1. A thin bed system relying on passive heat removal and evaporation of water from a relatively large surface area, as described by Smits *et al.*, (1998).
2. Forced aeration relying on an air stream to cool and evaporatively dry the substrate, as described by Lonsane (1992).

3. Agitation, relying on the mechanical re-distribution of the substrate and loss of heat and water from a constantly “renewed” surface.
4. A combination of (2) and (3).

In the first instance option (1) was rejected. This was seen as the least attractive option for a number of reasons:

1. Thin bed fermentation is inherently inefficient. Smits *et al.* (1998) describe experiments suggesting that in some systems enzyme production ceases below a bed depth of 5 cm, probably due to a limitation in efficiency of gas transfer. Gas transfer itself did not appear to be a significant problem in the present system, but thin bed fermentation probably requires a relatively long incubation time, compared to other methods, to achieve equivalent growth and metabolite production.
2. Thin bed fermentation requires relatively large areas of space for the trays, probably becoming excessive on scale-up, and in some systems can be very labour-intensive.
3. It is difficult to sterilise the substrate *in situ* without a very large autoclave; autoclaving the substrate separately then transferring to trays is unsatisfactory for systems that need to be maintained strictly aseptic.

4. It is difficult to maintain thin bed fermentations under strict aseptic conditions.

Sterility is not necessary for most of the “low-tech” applications thin bed fermentation is used for, but is considered essential for the present pharmaceutical type work.

Option (2) above, i.e. forced aeration, was considered to be attractive since the use of a forced air stream could potentially affect both drying and cooling simultaneously. Further, the well defined pore structure of the current substrate was considered to be ideally suited to this type of treatment, being likely to allow a relatively unhindered passage of air. In fact a type of aerated system had already been attempted in this project, and had demonstrated some drying effects. (Section 4.5.)

Ultimately, it was decided that the ideal system would combine aeration with some form of agitation (option (3) above), as previous studies in the project had indicated that the present system would tolerate at least intermittent agitation. It was anticipated that agitation would optimise the effects of aeration by re-distributing accumulated heat and water. Also, any tendency for the air to channel through the substrate would be alleviated by regularly turning the bed over. Agitation was also considered important in uniformly distributing the inoculum at the start of fermentation.

Bulgar wheat at an initial a_w level of 0.99 was used in this study for the following main reasons:

1. This is within the a_w range producing enhanced metabolite levels in previous studies.
2. Material at this a_w level is free flowing and therefore more “handleable”.
3. Material at this a_w level will resist the detrimental effects of water accumulation longer than initially wetter material.

The fermenter design was based around two 5 litre Duran bottles, one acting as the fermenter, and the other acting as the humidifier. The bottle type and size represented a simple form of scale-up from the previous study, which utilised 500 cm³ Duran bottles. It was decided to attempt a further scale change at this stage since the problems at previous scale were clearly identified and needed to be addressed at least at the scale attempted here. Unfortunately, removal of the substrate at the end of fermentation from the vessel was extremely difficult due to the very narrow neck, and this particular vessel type was not used in subsequent work.

In this study, substrate agitation was supplied by a separate bottle roller unit. It was necessary to separate the fermenter from the humidifier before rolling was carried out, and this was accomplished aseptically by the use of a double air filter system. Bottle separation was not an ideal arrangement, but appeared to be a simpler solution than attempting to design an internal stirrer system with the accompanying problems of maintaining sterility. In principle, this system was similar to a rotating drum bioreactor. A rolling type fermenter has also been described by Lindenfelser and Ciegler (1974). This was used for production of ochratoxin A by *Aspergillus*

ochraceus on cracked wheat. This fermenter was run continuously at a maximum speed of 16 RPM and achieved high production of the mycotoxin. However, the fermenter could not be autoclaved, and was instead sterilised using highly toxic ethylene oxide gas.

5.7.2. General observations.

This was not a successful study. Problems were encountered very early, with bottle rolling being shown to be entirely inadequate for substrate mixing after only 24 hr. Rolling was therefore replaced by manual shaking, although this was considered wholly unsatisfactory in terms of realistic method development. This problem was quickly followed by an obvious build-up of metabolic water, sufficient to severely inhibit fungal growth and pigment development. This was accompanied by an increase in temperature, indicating that heat build-up also was not under adequate control. The accumulation of metabolic water and heat indicated that the air stream was not working efficiently to control these parameters in the way intended. This may have been a simple consequence of an inadequate air flow with respect to the substrate volume involved, an air stream which was too damp to effect efficient drying (the humidifier unit contained pure water), or a combination of both of these.

A further major problem was the reappearance of bacterial contamination. Again, it was concluded that the contamination had been present from the start of the experiment. This was particularly disappointing since the autoclave cycle had been

increased to 50 min, and there seemed to be little scope to realistically improve the sterilisation efficiency beyond this.

The various problems encountered in this study were manifested in the metabolite profile obtained. The pattern of metabolites was highly atypical, and the levels produced generally low and variable. This result was anticipated due to the low growth and pigment development observed.

5.8. SECOND LABORATORY SCALE FERMENTER STUDY.

5.8.1. Method development.

The development of apparatus in this study was based on an effort to overcome the major difficulties experienced in the previous study. These may be summarised as:

1. Failure of forced aeration to effect drying and cooling in the fermenter, probably because the air flow was insufficient, or carrying too much moisture.
2. Bacterial contamination, probably arising from incomplete sterilisation.
3. Inability to mechanically mix the substrate after development of fungal growth.
4. Difficulty in emptying the fermenter due to the narrow neck of the vessel used.

Although forced aeration appeared to present the best solution for controlling moisture and temperature, it was necessary to make this more efficient. There was little scope for increasing the air flow rate significantly with the equipment available, due to the possibility of pressurising the system to the point of failure. At this stage the best

course of action seemed to be to attempt to use a much drier air stream, accomplished by passing the air through a glycerol / water solution, at a known a_w level, in the humidifier. This was accompanied by a modest increase in air flow rate.

With regard to the problem of bacterial contamination, it was felt that a major limitation in the autoclave apparatus used previously was the inability to monitor temperature within the substrate load, the autoclave cycle being controlled by fixed probes within the chamber. It was decided that further autoclaving was to be conducted at the premises of TerraGen Discovery Ltd., where apparatus was available to control autoclave cycles from probes which could be positioned in the centre of the fermenter. In fact an identical “dummy” fermenter was used so that the experimental fermenter was not disturbed. By effectively controlling the autoclave cycle from the centre of the substrate load, it was hoped that complete sterilisation of all the material present would be achieved.

The use of a submerged liquid fermenter for SSF work seemed an obvious choice. Stripped of its ancillary equipment it consists simply of a straight sided glass cylinder with circular stainless steel top and base plates which bolt into place using ring and seal assemblies. The use of such a vessel for SSF work offered a number of advantages:

1. The vessel is designed for repeated sterilisation cycles and can be assembled with the substrate *in situ* before sterilising.

2. The glass vessel allows the progress of the fermentation to be monitored visually.
3. The broad end profiles allow easy access for substrate addition and removal.
4. The top and base plate drillings allow easy fitting of ancillary equipment such as air lines and inoculation / sampling ports. Existing needle and septum fittings can be used in most cases.
5. The use of “standardised” apparatus such as this offers the opportunity for other workers to repeat, verify or further develop the work carried out here.

The only other reference which was found relating to commercially available equipment was the use of a Sartorius filter assembly as a solid substrate fermenter by Gumbria-Said (1996).

5.8.2. General observations.

The combination of increased flow rate and drier air appeared to achieve reasonable control of moisture level and temperature. However, there was a difficulty with local severe drying around the air entry points at the base of the fermenter. This was clearly due to the close proximity of air entry ports to the substrate base, and was not alleviated by the layer of glass wool below the substrate. The small accumulation of water on the glass walls and locally in the substrate indicated that there was still scope for improving moisture control, but this had to be balanced with the drying effects experienced elsewhere in the fermenter. The efficiency of air flow through the substrate was very satisfactory however, with virtually no drop in flow rate between the two rotameters.

The study was not subject to obvious contamination. The apparatus was successfully run for 5 days prior to inoculation and the system remained sterile. This was primarily to check the integrity of the system against the possible ingress of fungal contamination, since the specific bacterial problem seemed to be intimately associated with fungal development, and could not be expected to appear in an otherwise sterile system. Most importantly, no bacterial contamination was detected on culturing substrate samples following the fermentation. Clearly, it was not possible to screen the entire fermenter contents in this way, but the absence of culturable bacteria from the random samples tested probably indicates that the prior autoclaving of the substrate had been satisfactory on this occasion.

The inability to agitate the substrate was shown to be a disadvantage in this fermenter design. This prevented adequate distribution of the inoculum and subsequent fungal development was not satisfactorily uniform. This effect was further exacerbated by the local drying at the base of the fermenter. In fact, it would have been possible to manually mix in the inoculum by shaking, but this was seen to be inappropriate with a view to realistic method development.

5.8.3. Metabolite profiles.

The uneven nature of the fungal growth in the fermenter was reflected in the metabolite results obtained, which showed significant variations between top, middle and bottom samples. This is important since it indicates that the system was running heterogeneously and, more importantly, inefficiently. In spite of this, the levels of metabolites obtained in this study were generally very encouraging, with levels of

metabolite 1 (middle sample) exceeding those obtained previously. This was in marked contrast to the previous study where metabolite 1 had been reduced to a minor peak.

5.8.4. Other fermenter designs.

Glass column bioreactors with forced damp air aeration have been described by Dorta and Arcas (1998). They cultured *Metarhizium anisopliae* on a rice based medium and reported large variations in water accumulation, heat build-up and sporulation patterns within the columns. Durand *et al.* (1988) have described a statically incubated stainless steel fermenter of 2 kg capacity for protein enrichment of leached sugar beet pulp by *Trichoderma viride*. In this system a_w of the air stream was controlled by heating the humidifier water, which had the effect of increasing water uptake to approximately 100%. This system therefore ran at a far higher a_w level than the system used in the current work, but this seemed satisfactory for short fermenter runs of 48 hrs. The system developed by Durand *et al.* (1988) was not autoclavable. Almanza *et al.* (1995) have reported the development of a stainless steel bioreactor of 1 litre capacity with a similar forced aeration system that could be autoclaved before use.

5.9. THIRD LABORATORY SCALE FERMENTER STUDY.

5.9.1. Method development.

The final series of experiments were carried out with three main objectives in mind:

1. To attempt to demonstrate the possibility of mechanical stirring and to use this at least to distribute the inoculum uniformly.

2. To re-arrange the pathway of air through the system to minimise local drying.
3. To obtain reasonably uniform metabolite production within the fermenter.

In these studies, the initial a_w level of the substrate was reduced to 0.97 to attempt to minimise the effects of any water accumulation, and to optimise the “flowability” of the medium, in order to facilitate mechanical agitation.

5.9.2. Stirrer designs.

The development of an efficient method for mechanically agitating the solid substrate proved to be problematic. The first two arrangements (standard Bioengineering AG motor with standard and oversize Rushton impellers) failed because the motor (designed to stir liquids) did not have sufficient torque to turn the impellers through the solid. This problem was overcome by modifying the shaft to accept a standard workshop electric drill. This arrangement enabled the shaft to be turned, but the Rushton impellers (both standard and oversize) were unsuitable because they were unable to agitate the solid mass as a whole; instead they “tunnelled” a very small area within the breadth of their travel. The third arrangement involved production of three novel impellers designed specifically to run in a solid matrix. This arrangement worked well, at least with uncultured substrate, and virtually the entire mass could be “spun” at high velocity when the drill motor was run. In fact, the substrate particles appeared to become “air-borne” rather like a fluidised bed type system. However, subsequent attempts at mixing colonised material were less successful. The substrate presented high resistance to impeller movement and threatened to damage the drill motor. The substrate tended to break down into a number of large clumps, and the impellers moved between these rather than mobilising the substrate mass as a whole.

The major outstanding problem therefore appeared to be the difficulty in stirring the substrate efficiently during the fermentation. In fact, the results obtained in these experiments questioned the need for agitation of the substrate during fermentation, but it was generally felt that the ability to stir was advantageous for the following reasons:

1. Stirring would further promote homogeneous conditions in terms of heat dissipation, moisture distribution and uniform colonisation. These could become critical in any further scale-up.
2. Other systems, using different organisms and substrates may rely on stirring during fermentation for a successful outcome.
3. In some systems, stirring may stimulate particular secondary metabolite production.
4. Stirring may facilitate substrate removal at the end of fermentation.

The use of a workshop electric drill to operate stirrer equipment is not suggested as a final solution in developing an agitated SSF system. This represented a prototype arrangement which at least demonstrated the possibility of using electric motors in this type of application. The impellers also represented early designs which could be developed and improved with more work. An improved stirring arrangement could consist of multi-pronged rakes passing through the substrate slowly and turning it over like a plough. A high torque, low speed motor would be suitable for this and may represent a solution to mechanically agitating the colonised substrate.

5.9.3. Aeration – moisture and temperature control.

The re-orientation of the fermenter vessel through 90° to give a “tunnel” type arrangement was a very simple idea but significantly altered the profile of the substrate bed, making it shallower with a much increased free surface area. This arrangement facilitated a different air flow arrangement where entry air was routed into the headspace and passed through the substrate bed before exiting at the base. This arrangement successfully prevented local drying whilst permitting the use of extremely dry air in these two studies (intermittent unhumidified air in run 1, and continuous air set below 0.900 a_w in run 2). The reduced substrate depth also probably facilitated mechanical stirring since only part of the stirrer blade travel was in contact with the bed, i.e. the stirrers protruded above the surface and acted as “scoops”.

These fermenter runs may be considered to have been successful in terms of moisture and temperature control. The monitoring of temperature of external glass at one location was obviously a crude method, but it was not possible to include the internal temperature probe as had been used in the previous study, due to the inclusion of the stirring apparatus. In spite of this crude method, the improved temperature control observed in run 2 compared to some earlier studies was unequivocal.

Sintered glass gas bubblers were used as air entry and exit ports in these studies as it was felt that they would be less likely to block than the needle type fermenter fittings. In fact they appeared to perform very well, and the layer of glass wool that had been used previously to help prevent blockages was omitted from the system.

5.9.4. Bacterial contamination.

Bacterial contamination appeared to be absent from both runs 1 and 2, and no culturable bacteria were obtained from samples plated out after the fermentations. Run 2 was further protected by the inclusion of chloramphenicol. The presence of this antibiotic did not seem to influence the fermentation in any other way and most interestingly it did not appear as an obvious additional peak in the any of the HPLC traces. The apparent absence of bacterial contamination in run 1 (without chloramphenicol) probably indicated that the autoclaving process was again successful, although it is possible that the relatively low a_w conditions that were maintained in these studies could be sufficient to inhibit bacterial growth.

5.9.5. Metabolite profiles.

Metabolite profiles produced in both of these studies were very encouraging. In run 1, comparatively high levels of metabolites were produced after only 7 days and, perhaps more importantly, extremely uniform levels were obtained from the three sampling regions. This represented a marked improvement on the previous study where significant differences between fermenter locations were observed. Run 2 showed greater, and statistically significant variations in metabolites 1, 2 and 3 for the two sampling positions. However variations in metabolites 1 and 2 were at least smaller than for the previous unstirred fermenter experiment.

It is interesting to compare the development of metabolites in these two fermenter runs with each other, and also with the relevant treatments in the temporal experiment (Section 4.4.). Metabolite 1 showed more advanced production at day 7 in the present studies although levels rose higher in the temporal study by day 14. Metabolite 2 and its twin appeared by day 7 in run 2 but were absent from run 1. In this respect run 1 mirrored the temporal study more closely in that in the latter these metabolites did not appear until after day 7. Day 7 levels of metabolite 3 were comparable for all three studies. This metabolite then showed an increase by day 14 for run 2 which was not mirrored in the temporal study, where a decline was noted beyond day 7. In general, these results continue to demonstrate the variability of metabolite responses under apparently similar conditions.

5.9.6. Other stirred fermenter designs.

A number of SSF fermenter designs incorporating substrate agitation are described in the literature; Durand *et al.* (1993) have described a large scale aerated and agitated bioreactor mainly intended for protein enrichment of animal feed. This had a working volume of 1.6 m³ and substrate bed depth of 1 metre. The system utilised ethylene glycol in the humidifier water to control a_w of the air stream. The agitation device consisted of a conveyor with three vertical screws which moved back and forth across the top of the reactor. Although not stated, it seems unlikely that this large scale system ran strictly aseptically. Horizontally oriented bioreactors with aeration and agitation via motor driven paddles have also been reported by Durand *et al.* (1996). These were not designed to run aseptically. A further stirred design has been reported

by Chamielec *et al.* (1994). They developed a stainless steel bioreactor of 50 litre capacity with a “planetary motion” agitation device powered by an electric motor.

Overall, this project has demonstrated the feasibility of scaling-up a solid substrate system to a level that would normally be considered pilot scale. This was achieved by careful selection of a suitable fungus/substrate model system, and application of ecophysiological relevant stresses, to produce a favourable metabolite response. Although the conditions imposed in this case would not be appropriate for all fungal species, the methods used were not prohibitively restrictive. In general, attention to fungal ecology and appropriate environmental pressures is advocated as a valuable approach to secondary metabolite, or, natural product, discovery and development.

The fermentation system used was based, for the most part, on “standard” equipment, readily facilitating further development and validation. The approach of “trial and error” in scaling-up a solid substrate system was shown to be a valid, if frustrating, development method.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.0. CONCLUSIONS.

6.1. Work with a *Phoma* species.

Work with the *Phoma* species, using homogeneous extracts of a range of agricultural materials, was designed to be preliminary to work with solid substrates proper. Growth and squalstatin S1 production were compared for a rich medium (malt extract agar (MEA)), and various nutritionally depleted grain and seed extract media over a range of temperatures and a_w levels.

The growth of the fungus was shown to be strongly influenced by temperature in the range 15-25°C, with maximum growth rate at 25°C, and only mildly influenced by a_w in the range 0.998-0.960. In marked contrast to this, S1 production was strongly influenced by a_w , with maximum production in the range 0.990-0.980, and strongly inhibited at 0.998 a_w . For example, at 25°C, levels of S1 produced in wheat extract at 0.980 a_w were 10x greater than at 0.998 a_w . Overall, production of S1 was not influenced by temperature fluctuation in the range employed. S1 production was strongly influenced by medium type, maximum production being observed in wheat extract in most experiments. For example, at 25°C, levels of S1 produced in wheat extract at 0.980 a_w were approx. 8x greater than oil seed rape extract, 5x greater than malt extract and 2x greater than oat extract.

In general, these studies highlighted the importance of the manipulation of environmental parameters in secondary metabolite production, and in particular the

role of stress (see Section 6.4.). This work also served to suggest the types of substrates that could be usefully employed in subsequent work in the project.

6.2. Solid substrates.

A number of solid substrates were used in the course of this project, and moisture sorption isotherms were prepared for some of these for the first time. Overall, the most suitable substrates for growth and secondary metabolite production appeared to be wheat and wheat products. Similar findings have been reported in a number of previous studies that appear in the literature. A processed form of wheat (bulgar wheat) was ultimately chosen for development of a fermentation system. This substrate had a number of important attributes which are listed in Section 5.3.5. A tentative list of desirable properties for solid substrates used in this type of fermentation work was also drawn up (Section 5.2.1.).

6.3. Development of a fermentation system.

The development of a fermentation system in this project adopted the approach of “trial and error” as suggested by Lonsane *et al.* (1992). This involved devising a system around simple scale-up steps from first principles, and addressed problems as they appeared. The project demonstrated the feasibility of scaling-up the system at least to the level of approx. 3 litres of wet substrate, from initial studies working with single volumes of 40 cm³ wet substrate. This represents an overall volume increase of x 75.

The apparatus used in the project was essentially submerged liquid fermentation equipment with a number of modifications, an approach which has not been reported previously. In particular, the use of such “standard” equipment allows for direct validation and development work by other researchers. The final design obtained represents an early prototype with much scope for further development and improvement.

Of the three main metabolites followed, highest titres were not always achieved in the final fermenter studies when compared with all the studies conducted. However, significantly more metabolites were always produced in fermenter studies compared to initial small scale studies with the same substrate (bulgar wheat). For example, for metabolites 1 and 3, approx. 17x and 3x increases in levels were achieved in the second horizontal fermenter run, and metabolite 2 was absent from the small scale study at the relevant a_w level. Further, fermenter studies demonstrated more rapid production of some metabolites (e.g. metabolite 1) compared to earlier experiments.

6.4. The role of environmental stress.

In this project, there was clear evidence that metabolite production responded to stress. Stress effects included imposition of low a_w and, particularly in the case of the *Phoma* study (where nutritionally rich and depleted media were compared), low nutrient conditions. Often, varying metabolite responses were noted under conditions

that had no significant effects on growth. This was clearly demonstrated in the initial work with *Phoma* species, where squalastatin production was strongly influenced by slight alterations in a_w , and nutrient depletion (see section 6.1.), and also in the SSF work with *E. nigrum*, where relatively minor alterations in a_w elicited markedly different metabolite responses. For example, in small scale studies with medium A, 5 unique metabolites were obtained at lowered a_w levels. Further, 2 metabolites that were represented at high a_w , eluting at 2.6 and 6.4 min, were increased by 14x and 20x respectively at 0.98 a_w . In fact, stressing the fungi in a *subtle* way was considered key in this work. This was in contrast to much previous published work concerned with secondary metabolite production under conditions of reduced a_w , where very harsh conditions have often been imposed, so not surprisingly, inhibition of metabolite production was typically observed.

Another key point seemed to be the *relevance* of the stress effects to the organism in question. This was demonstrated by the example of the different responses of *E. nigrum* and *S. palmicola* to lowered a_w , and suggested that the type of stress imposed needed to be relevant to the lifestyle of the organism to effect a stimulatory metabolite response. Such responses therefore represent adaptations of the organism to changes in pertinent environmental conditions, and the experimental manipulations represent simulations of environmental conditions that the organism could be expected to encounter in its normal “lifestyle”. It is reasonable to assume that such mechanisms would evolve in successful organisms, and this further underlines the competitive role of secondary metabolism for survival. Further, it is suggested that attention to the ecophysiology of producer organisms may have benefits in natural products screening

programs, both in terms of metabolite levels and ranges. This also reflects the modern “chemical ecology” approach to natural products discovery.

In general, the approach of deliberately stressing the organisms contrasts with the normal methods of laboratory culture. This work suggests that culturing these fungi species on rich, homogeneous laboratory media is not the best way to exploit their biosynthetic potential.

6.5. Metabolite production patterns.

The project has demonstrated the advantages of the use of low a_w conditions, both in rendering the solid substrate more manageable in scale-up systems, but also in the production of enhanced metabolite levels. In the case of the work with *E. nigrum*, this was perhaps a slightly artificial situation where an organism specifically capable of high metabolite production under conditions of low a_w was deliberately selected. In fact, *E. nigrum* was shown to produce optimum levels of most key metabolites in the range 0.99-0.98 a_w .

It has been fascinating to observe the sometimes intermittent and erratic appearance of metabolites in these studies, not only in experiments where conditions have been deliberately altered, but also (more frustratingly), under conditions that have been maintained as stable as possible. Perhaps this has been a consequence of the inherent heterogeneity of the systems used here, and the heterogeneity of the fungi themselves,

when viewed as dynamic, responsive systems. Metabolites 1,2 and 3 in *E. nigrum* could be produced reasonably reliably, but other metabolites (in all fungi studied) often seemed transient and elusive. From this work, secondary metabolite production can be viewed as an extremely delicately balanced process where a whole array of outcomes are elicited by subtle perturbation of environmental conditions. This is, again, evidence for the “survival” function of secondary metabolism, assuming an extrinsic biological function of the products. In effect, these experiments can be taken to represent very crude simulations of the multiplicity of conditions provided by nature and the strategic responses of the fungi.

6.6. This work in context.

To place this work in context it is necessary to consider how the pharmaceutical industry is most likely to use SSF as a tool for drug discovery and production. A new drug lead discovered in an SSF system is likely first to be assessed for the possibility of synthetic production. This is because, if feasible, synthetic production will almost always be cheaper and simpler. If the synthetic route is not possible, a SLF method may be considered, where a vast body of knowledge and sophisticated equipment is available. However, there are many fungi that cannot be cultured in SLF systems, and so SSF may finally be the only realistic option. This would represent a unique situation and would present a number of difficulties. The greatest problem is likely to be satisfying the requirements of Good Manufacturing Practice (GMP), to which all drug manufacture is subject. This requires full traceability of all aspects of production

and a high degree of process control and reproducibility. Reproducibility in particular continues to be a problem in SSF.

In the first instance, apparatus such as that developed in this project is more likely to be applied to discovery and development work rather than commercial production. This type of fermentation capability would be particularly useful for development work on new drug leads, for example to produce enough of a compound for structural elucidation. In applications such as this it would represent a quicker and more efficient route than conducting multiple small scale jar experiments, as is usually practised by the sponsor company currently.

6.7. Future work.

1. Further studies with the existing system to test overall reproducibility.
2. Studies with other fungi and/or substrates to determine the “robustness” of the system.
3. Further development of the agitation system to achieve mixing of the colonised substrate during fermentation to improve uniformity of growth and metabolite production.
4. Investigation and possible screening of some of the metabolites obtained in this project under low a_w conditions, as candidate drug leads.
5. Improvement in monitoring of critical parameters during fermentation, to include temperature analysis at a number of points, gas analysis and continuous a_w analysis. Possible link-up to a computer or data logger for continuous monitoring.

6. Further scale-up, perhaps initially to approx. 10 litres wet substrate volume.
7. Investigation of dynamic, fluctuating systems where conditions such as temperature, nutritional status, water content and gas balance are altered during the course of the fermentation.

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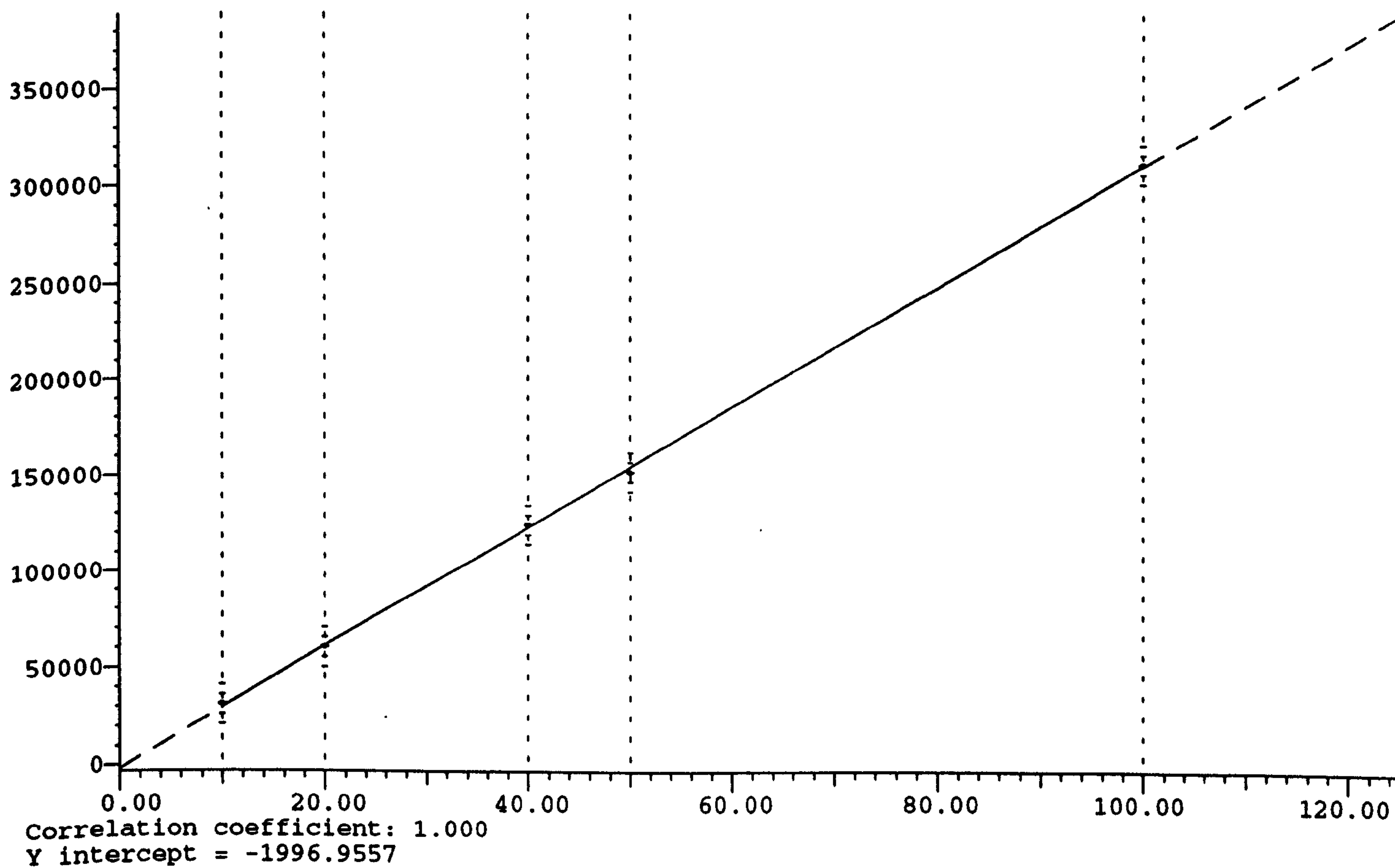
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APPENDIX I

***PHOMA* STUDY – HPLC CALIBRATION CURVE AND METABOLITE EXTRACTION EFFICIENCY**

----Calibration Plots of files in: C:\GILSON\DAVE.USR\SQUAL.014----
Analysis Method: C:\GILSON\DAVE.USR\SQUAL.MTI
using a Linear Regression fit

S1



Extraction efficiencies for recovery of squalestatin S1 from agar matrix.

Method 1.

MEA: 71.8 %

WEA: 77.6 %

Method 2.

MEA: 71.2 %

WEA: 73.6 %

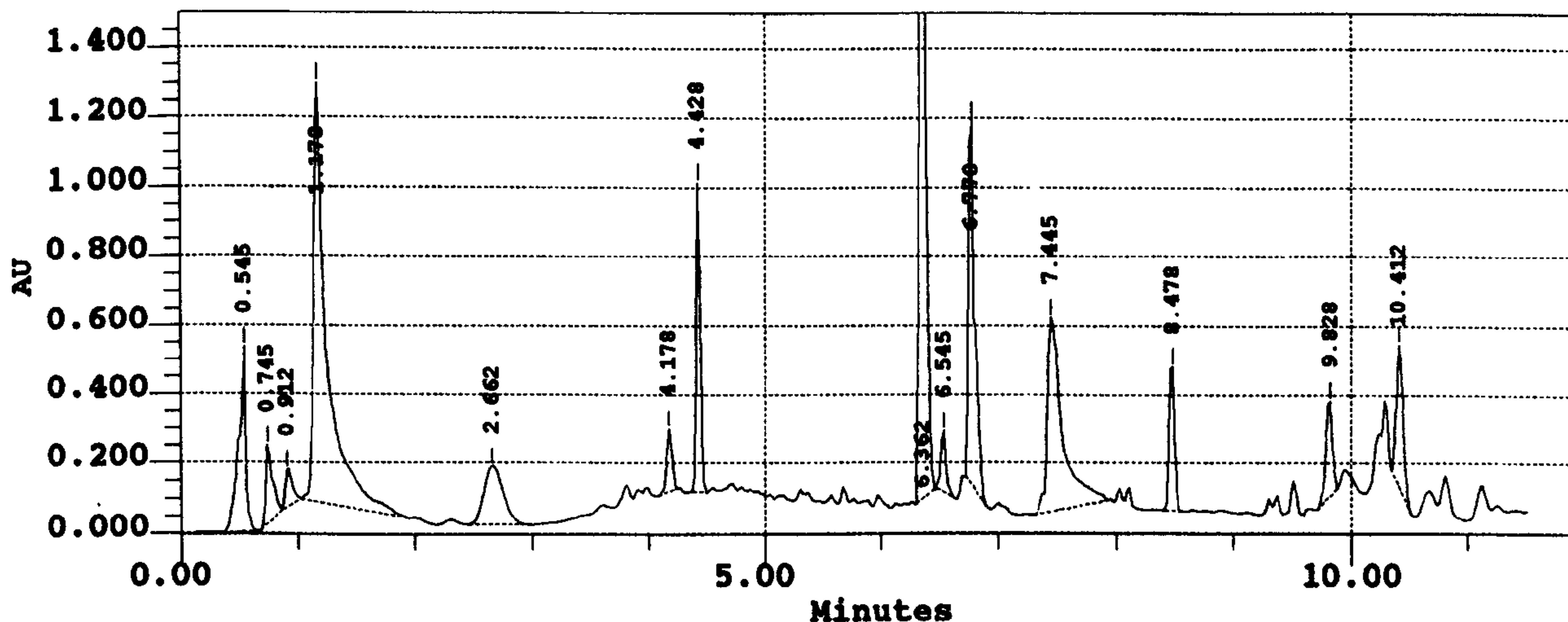
OEA: 67.1 %

OSREA: 75.7 %

APPENDIX II

SOLID SUBSTRATE STUDIES – EXAMPLE OF HPLC METABOLITE REPORT

SampleName: B17	SampleSetName: Cranfield_DA	Page 1 of 2
XenovaName: Cranfield Project	2020ProjName: PriFerm	Vial: 65
Channel ID: 10051	Acq System: mcr11	Volume: 15.00
ProjectCode: B-021	Acq Meth Set: 717_46_StdA_JP_msInj: 1	
Date Acquired: 20/03/98 07:55:10	Proc Method: Std_46_pm	
Date Processed: 20/03/98 08:10:32	ColumnID: T72811 V09	
Wavelength Processed: PDA MaxPlot (200.0 nm to 600.0 nm)	Initials: JP	

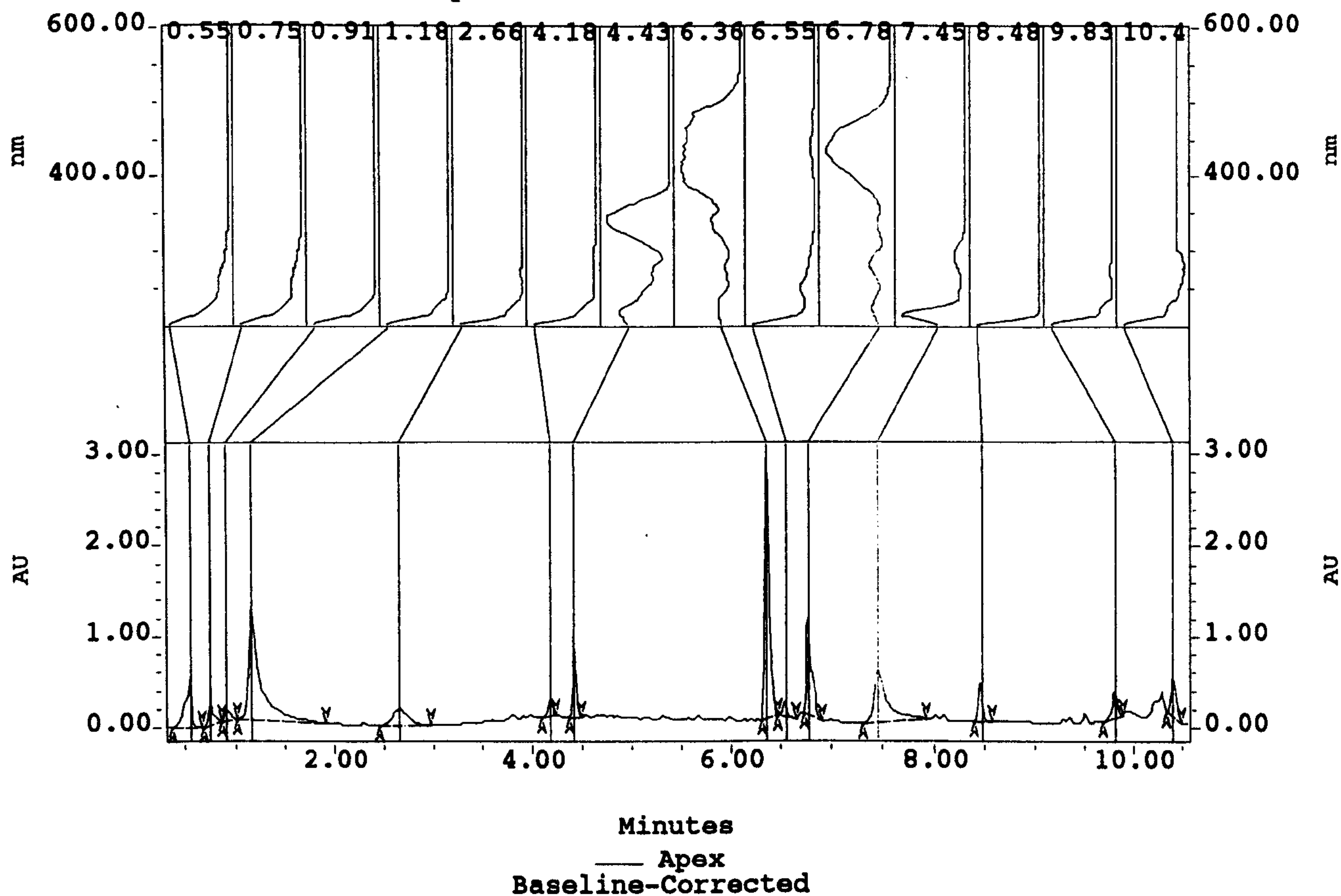


Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		0.545	2137540	535127		BB
2		0.745	860043	223786		BB
3		0.912	398119	103997		BB
4		1.178	9758420	1209307		BB
5		2.662	1866207	164412		BB
6		4.178	550781	180072		BB
7		4.428	2128206	896104		BB
8		6.362	10826229	2844970		BB
9		6.545	568518	179408		BB
10		6.778	3932193	1056714		BB
11		7.445	5030705	564602		BB
12		8.478	1372808	418619		BB
13		9.828	1098826	275102		BB
14		10.412	1635928	411963		BB

SampleName: B17	SampleSetName: Cranfield_DA	Page 2 of 2
XenovaName: Cranfield Project	2020ProjName: PriFerm	Vial: 65
Channel ID: 10051	Acq System: mcr11	Volume: 15.00
ProjectCode: B-021	Acq Meth Set: 717_46_StdA_JP_msInj: 1	
Date Acquired: 20/03/98 07:55:10	Proc Method: Std_46_pm	
Date Processed: 20/03/98 08:10:32	ColumnID: T72811 V09	
Wavelength Processed: PDA MaxPlot (200.0 nm to 600.0 nm)	Initials: JP	

Spectrum Index Plot Peaks 1 to 14



APPENDIX III

POSTER PUBLICATIONS

Interaction of nutrient substrate and environmental factors on secondary metabolite production by a *Phoma* sp.

D. Aldred, N. Magan and B. S. Lane*

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INTRODUCTION

- ◆ The pharmaceutical industry still relies heavily on microbial secondary metabolites as a source of new drugs for important clinical conditions.
- ◆ New products are obtained in large scale drug development programmes involving screening of thousands of compounds and extracts.
- ◆ At present little attention is given to the ecophysiology of producer organisms, and its effect on secondary metabolite production.
- ◆ The present study investigated the effects of nutrient substrates and environmental factors on temporal growth and production of a pharmaceutically useful secondary metabolite in a fungal model system.

MATERIALS AND METHODS

Fungus: *Phoma* sp. producing a squalenstatin (S1). Squalenstatin is a type of zaragozic acid, a class of secondary metabolites that have been found to be potent inhibitors of mammalian cholesterol synthesis.

Nutrient substrates: Malt extract agar (MEA), wheat extract agar (WEA), oat extract agar (OEA) and rape seed oil agar (RSOA). MEA represented a rich medium while the grain extracts represented nutritionally poor media.

Environmental factors:

- Temperature:** Experiments were carried out at 15, 20 and 25°C.
- Water availability:** Experiments were carried out with media at water activity (a_w) levels of 0.998, 0.995, 0.990, 0.980, 0.960 modified with the compatible solute, glycerol.

Growth studies: Agar plates were inoculated centrally with 4mm seed plugs. Growth was followed by measurement of colony radius for up to 30 days.

Secondary metabolite studies: Plates were destructively sampled for secondary metabolite analysis at 10 day intervals by removal of 4mm agar plugs from colonies in a pre-determined pattern. Plugs were extracted in acidified 80% acetonitrile:water for 1 hour then analysed by HPLC against a calibration curve of S1. (Mobile phase: acidified 55% acetonitrile:water).

RESULTS

Growth

Figure 1 illustrates the typical effect of a_w on temporal growth. Growth tended to increase with increasing a_w , but was only significantly reduced at 0.96. No significant reduction was noted for MEA at 25°C.

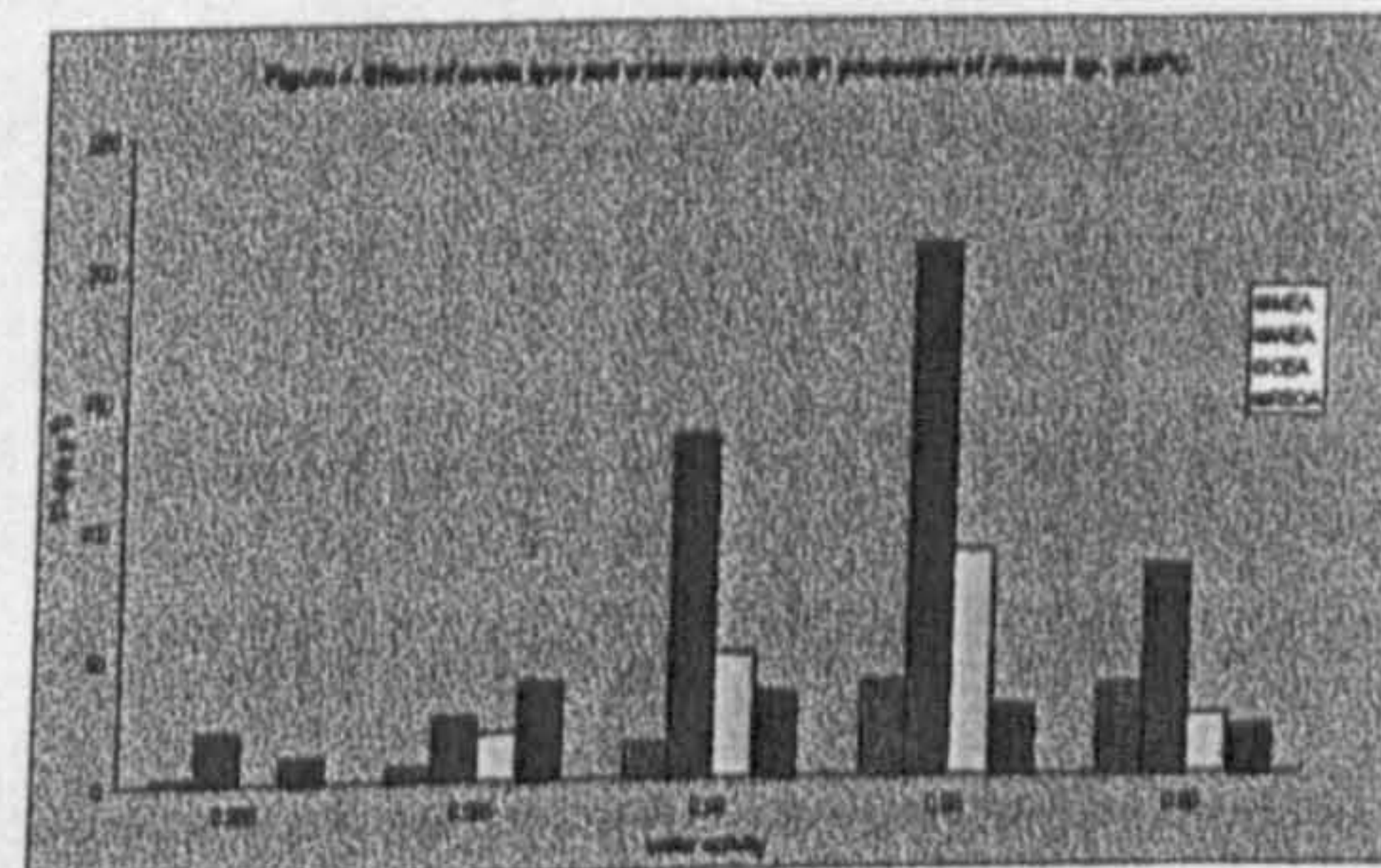
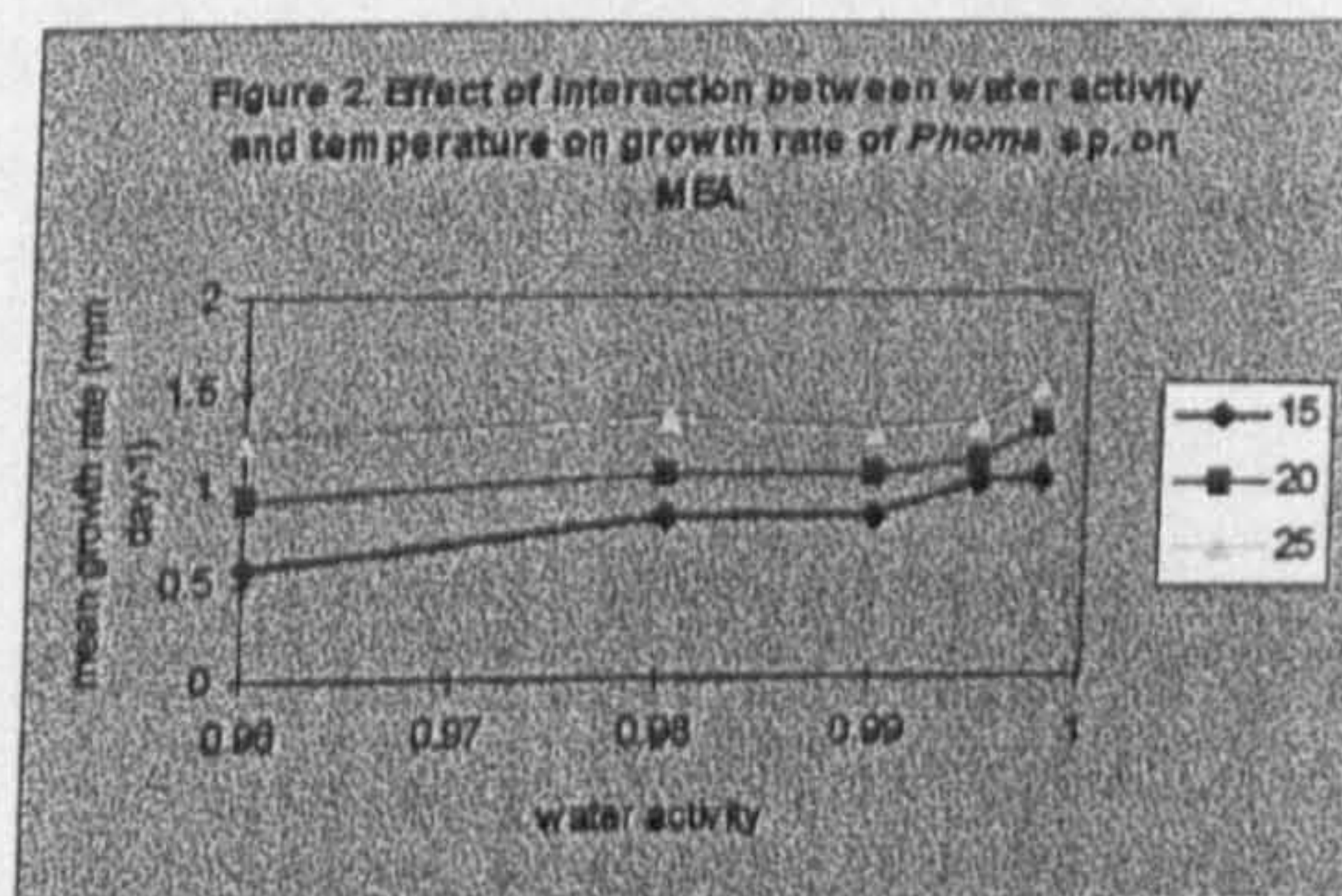
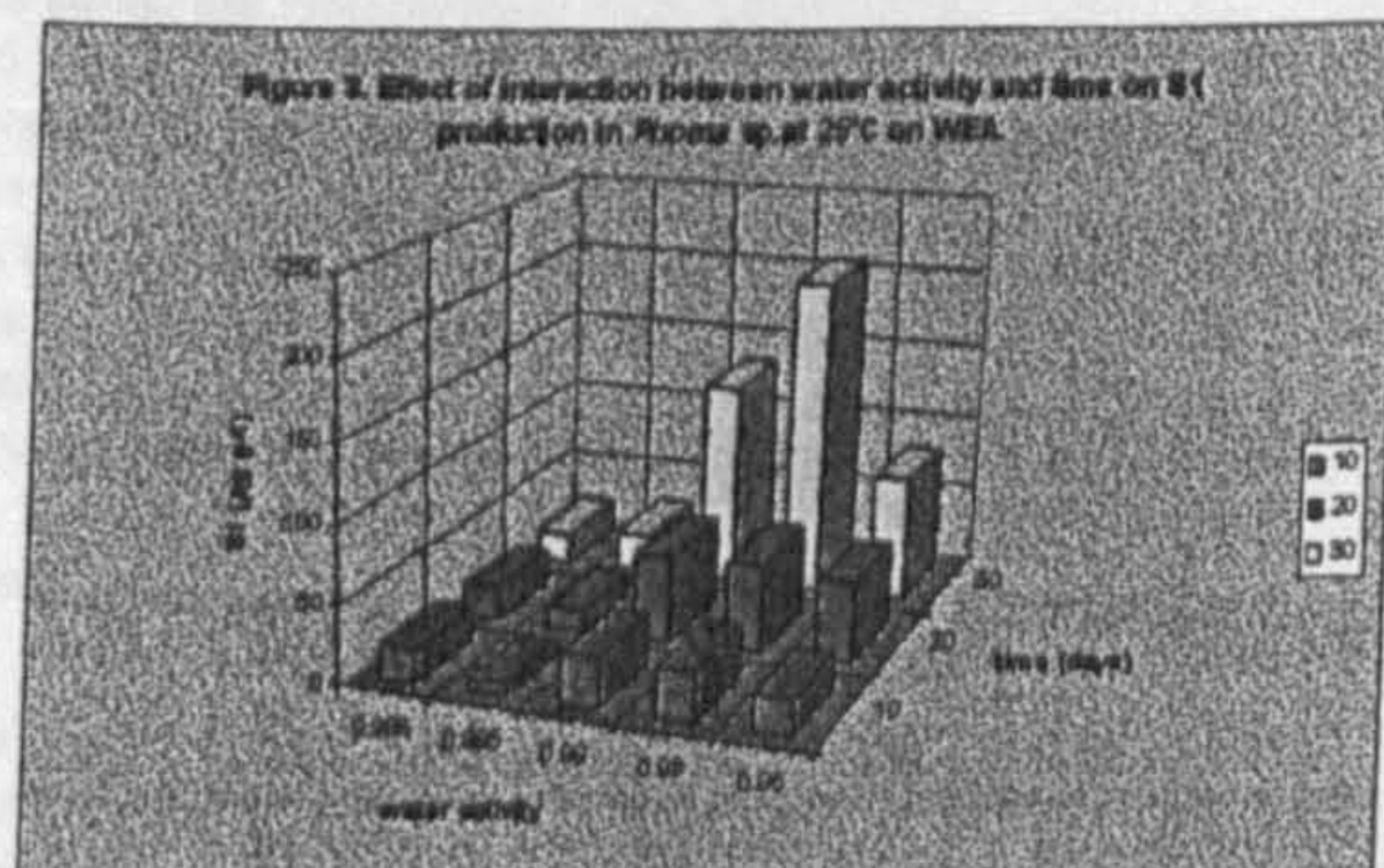
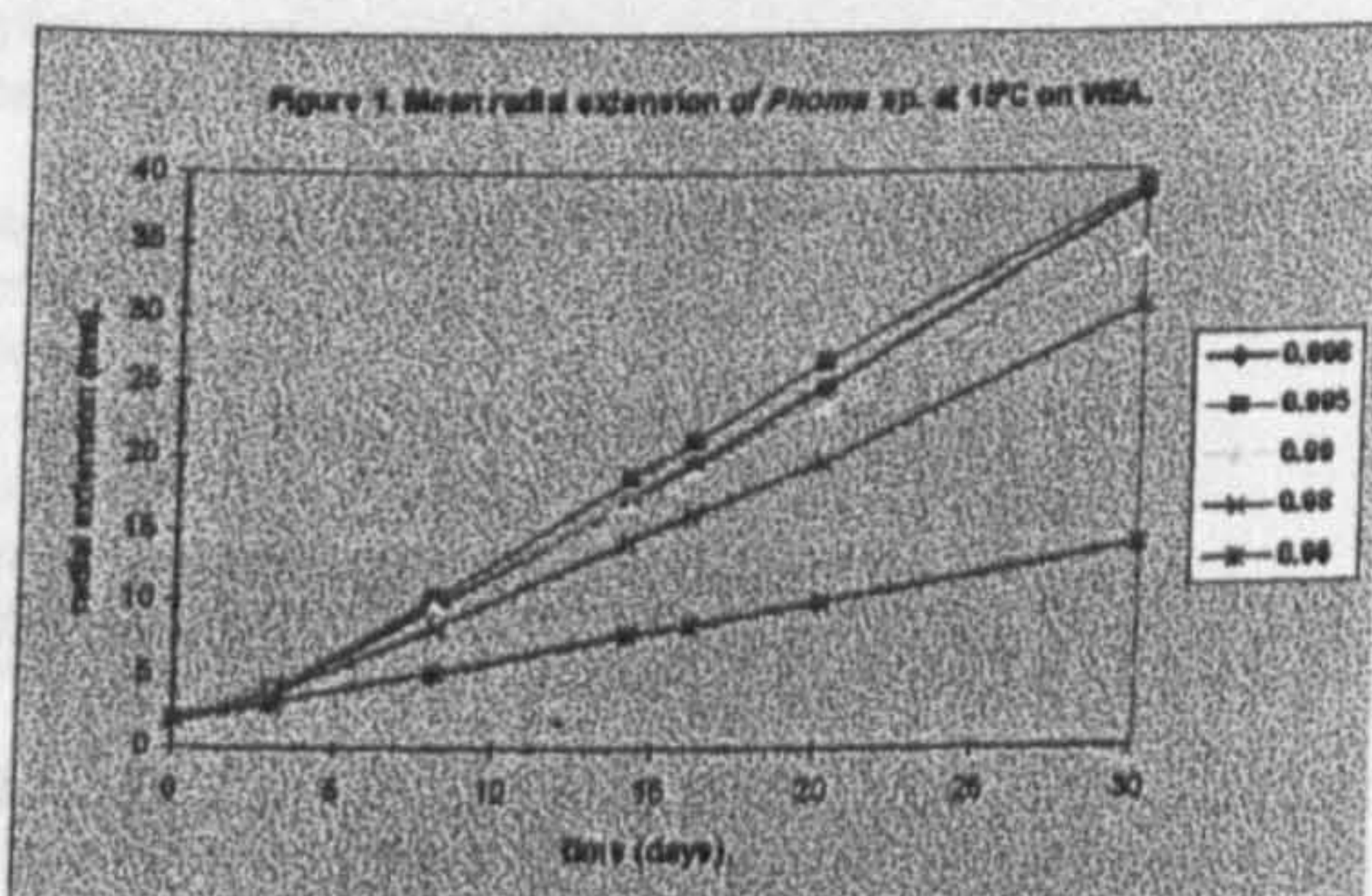
Figure 2 shows the effect of the interaction between temperature and a_w on growth rate for MEA. The effect of temperature on growth rate was more significant than a_w over the range employed.

The nutritionally depleted grain media induced a "rapid/effuse" growth form while the richer MEA induced a "slow/dense" growth form.

Secondary metabolite production

Figure 3 shows the interaction between time and a_w on production of S1 at 25°C on WEA. This shows a typical response with accumulation of metabolite over 30 days and maximum production at 0.99-0.98 a_w .

Figure 4 shows levels of S1 production as a function of a_w at 25°C for all media types. WEA produced maximum S1 at this temperature with notably earlier production than that on MEA.



CONCLUSIONS

- ◆ This study has shown that nutritionally depleted substrates may be usefully employed in the production of some secondary metabolites.
- ◆ Secondary metabolite production in this fungus responded strongly to manipulation of water availability within a range that was slightly inhibitory to growth. High a_w was inhibitory to secondary metabolism.
- ◆ Growth of this fungus was markedly influenced by temperature while secondary metabolism was influenced strongly by a_w .
- ◆ This study suggests that attention to fungal ecophysiology in screening programmes may lead to significant improvements in titres of secondary metabolites. This approach may also lead to a wider range of useful secondary metabolites being produced.

Interaction of nutrient substrate and water activity on secondary metabolite production by *Epicoccum* sp.

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Introduction

- The pharmaceutical industry still makes use of microbial secondary metabolites as a source of new drugs for major clinical conditions.
- New products are obtained in high throughput drug discovery programs involving screening of thousands of extracts and compounds.
- Microbial compounds are obtained under "standard" conditions often with little consideration of the natural ecophysiology of the producer organisms.
- In particular, the use of solid matrices, the natural environment of most fungal spp. is a largely neglected area.
- The present study investigated the effects of manipulating solid nutrient substrates and water availability on the secondary metabolite profile of an *Epicoccum* sp.

Materials and methods

- Fungal isolate:** *Epicoccum* sp. from the culture collection of Terragen Discovery Ltd. with an established secondary metabolite profile on a solid medium.
- Nutrient substrates used in the study:**
 - Substrate A: developed by Terragen Discovery Ltd.
 - Substrate B: whole wheat grain / perlite 3:1 (vol.).
 - Substrate C: whole oat grain / perlite 3:1 (vol.).
 - Substrate D: whole rape-seed / perlite 3:1 (vol.).
- Water availability:** Solid media were set to water activity levels of 0.998, 0.990, 0.980 and 0.960 by precise addition of reverse osmosis water with reference to moisture sorption isotherms previously constructed for each medium type.
- Experiments were carried out in 100 cm³ jars with vented lids. Jars were filled to constant volume. Experimental treatments (3 replicates) were placed in sealed transparent boxes with humidity control.
- Inoculation:** Each jar was inoculated with 0.2 cm² of a hyphal fragment inoculum derived from 2 week malt extract agar plates incubated at 25°C.
- Temperature and time:** Experiments were carried out at 25°C for one week and 18°C for a further week (total duration 2 weeks).
- Secondary metabolite analysis:** The contents of all jars were extracted and analysed using HPLC following the methods of Terragen Discovery Ltd.

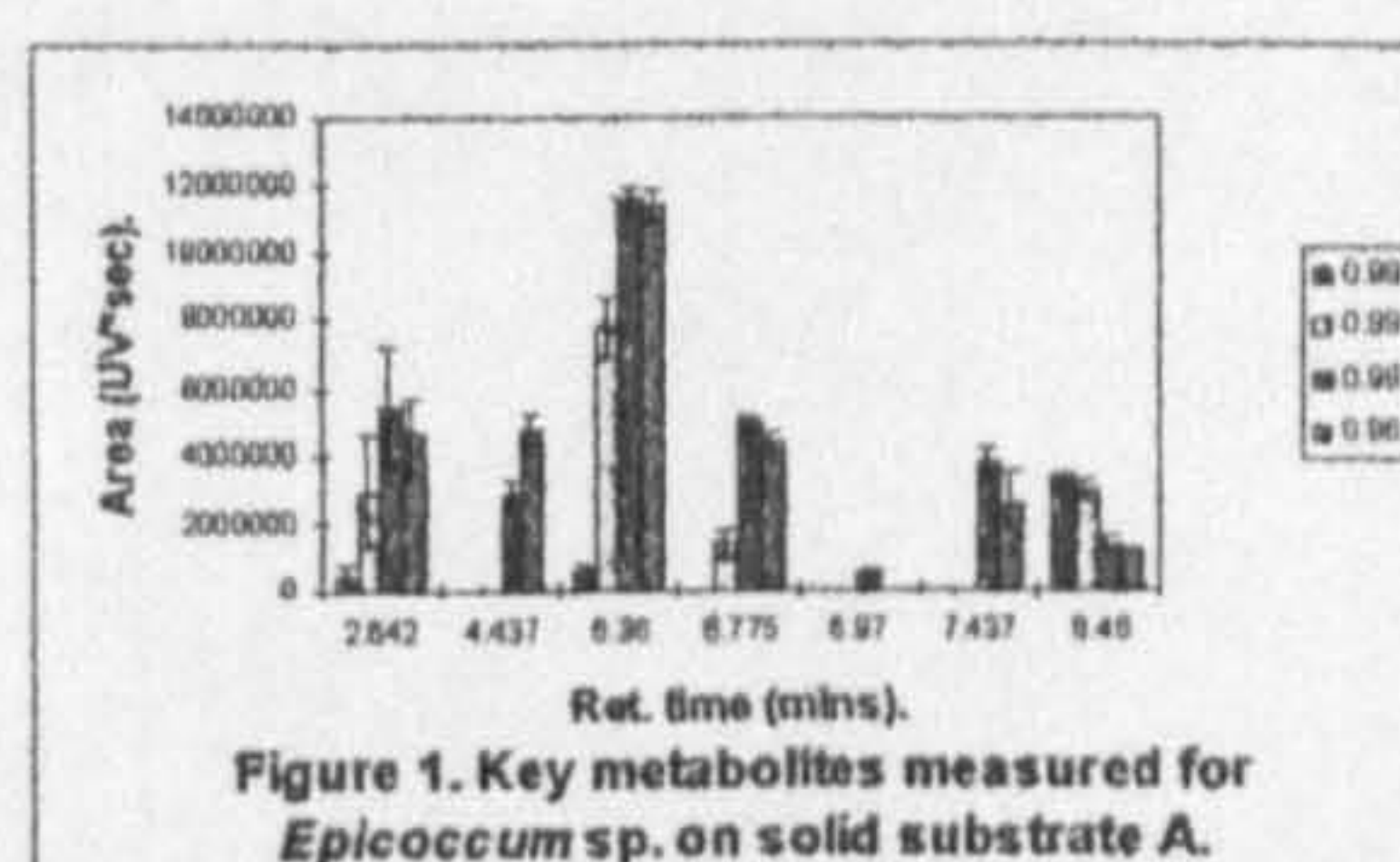


Figure 1. Key metabolites measured for *Epicoccum* sp. on solid substrate A.

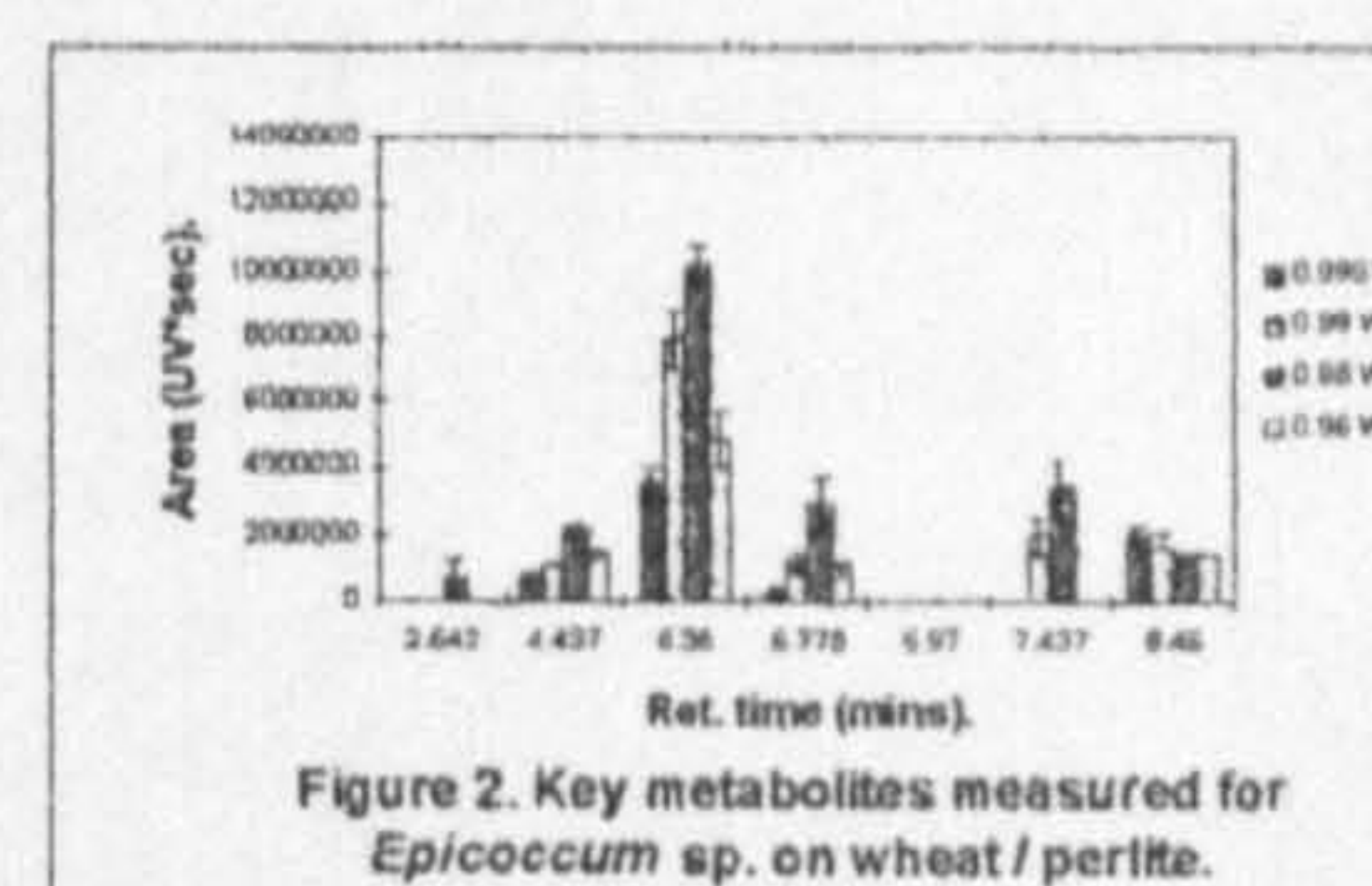


Figure 2. Key metabolites measured for *Epicoccum* sp. on wheat / perlite.

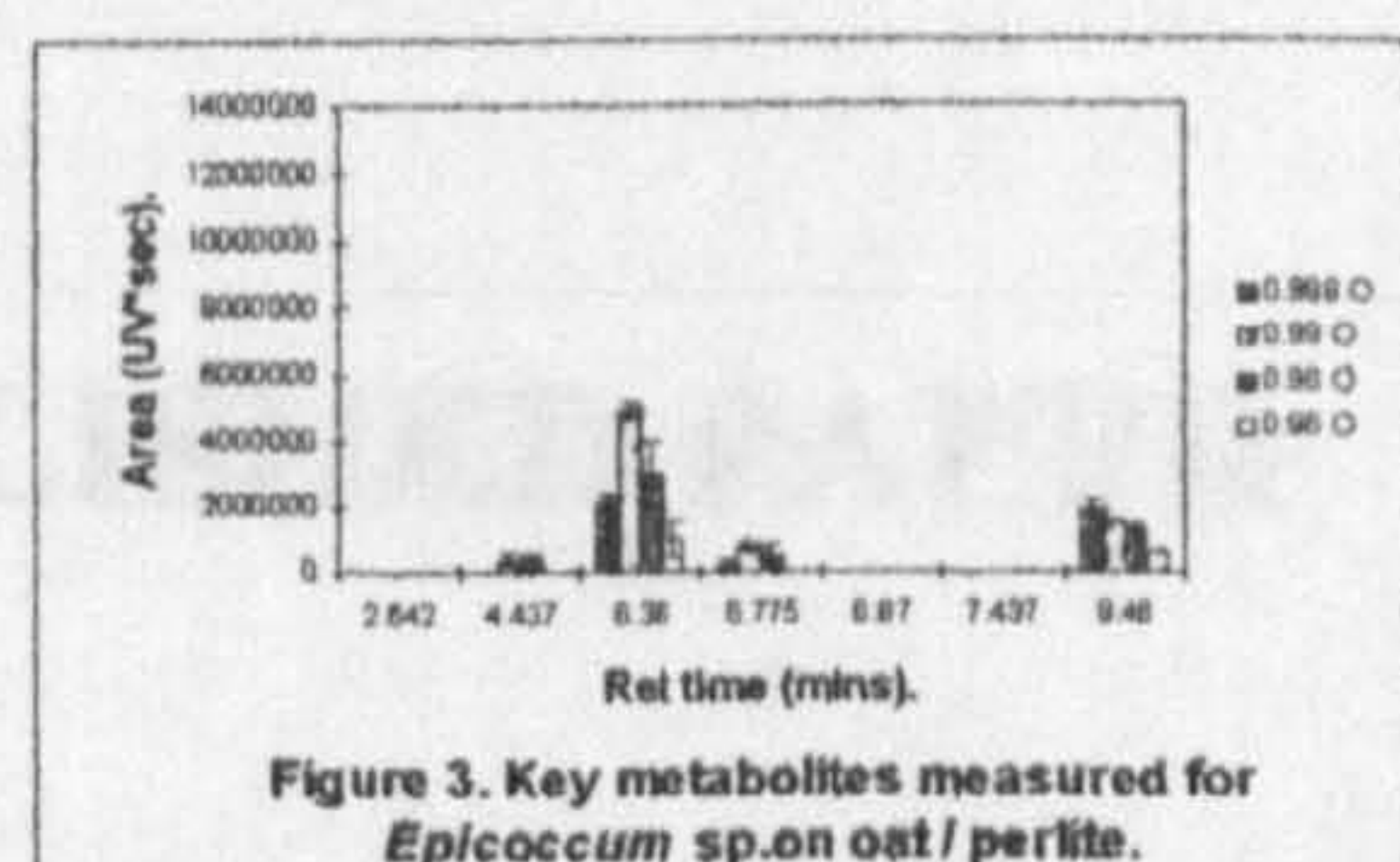


Figure 3. Key metabolites measured for *Epicoccum* sp. on oat / perlite.

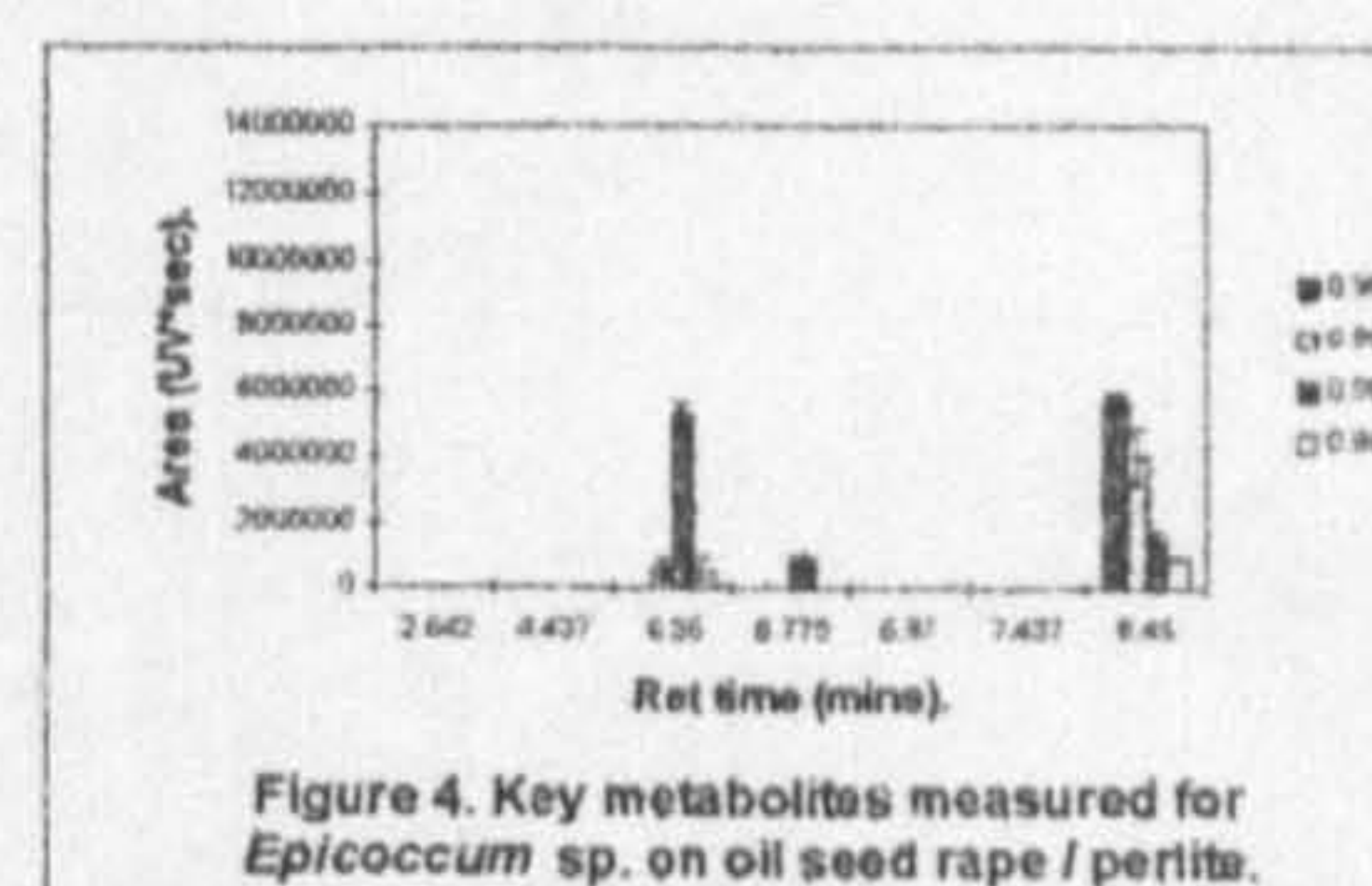


Figure 4. Key metabolites measured for *Epicoccum* sp. on oil seed rape / perlite.

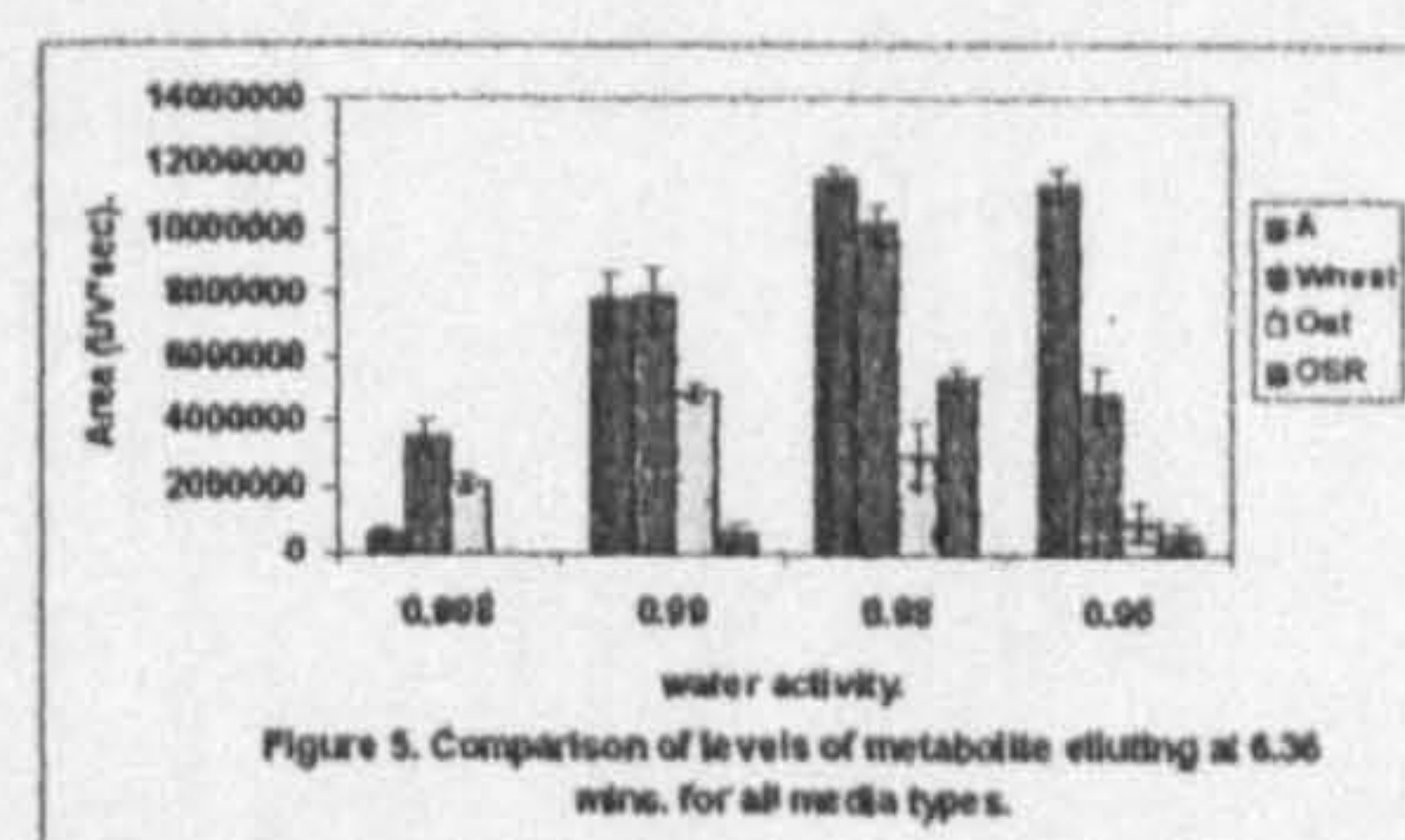


Figure 5. Comparison of levels of metabolite eluting at 6.36 mins. for all media types.

Results

Effect of substrate type on secondary metabolite profiles.

Figs. 1-5 show metabolite profiles of *Epicoccum* sp. for substrate A, wheat grain, oat grain and rape-seed respectively (Fig. 5 compares levels of one metabolite for all media types). It is clear from these figs. that substrate type had a significant effect on the range and levels of metabolites produced by the fungus. Fig. 1 indicates that substrate A produced both the widest range of metabolites and the highest levels. This is in fact a relatively complex medium consisting of three major ingredients in addition to some minor supplements. Of the relatively simple grain/seed media, wheat grain produced the best results with a similar metabolite profile overall to substrate A but consistently lower levels. Oat grain produced a far more restricted range of metabolites and markedly lower levels compared to wheat. Rape-seed was the poorest performer with the most restricted range of metabolites at levels similar to oat grain. Of the two starchy grains (wheat and oat) oat probably performed less well due to the presence of a fused glume outside of the fruit coat which constitutes a tough "husk". Wheat lacks this outer layer which probably renders it more accessible to utilisation by the fungus. Rape-seed, an oily substrate, was clearly unsuitable for this fungus.

Effect of water availability on secondary metabolite profiles.

It is clear from Figs. 1-5 that metabolite production responded strongly to the manipulation of water availability within the range employed. Most significantly all substrate types produced metabolites at decreased water availability levels that were absent under conditions of free water (0.998 a_w). In addition, with the exception of one metabolite (eluting at 8.46 mins.), all metabolites were produced at increased titres for water activity levels below 0.998. Overall maximum metabolite levels were observed in the range 0.99-0.98 for wheat, oat and rape-seed and 0.99-0.96 for substrate A.

Conclusions

- Secondary metabolite production in this fungus was strongly influenced by substrate type.
- Manipulation of water availability conditions resulted in a strong response in both secondary metabolite range and levels. More metabolites at consistently higher levels were produced at significantly lower water activity levels.
- This study has suggested that attention to fungal life-style and natural ecophysiology may enhance new lead molecule discovery in drug development programs.

Further work

This study investigated key metabolite levels at a single time point (i.e. at two weeks incubation). Future work will look at metabolite development patterns with time, using the same fungal isolate and similar experimental parameters.

APPENDIX IV

PUBLISHED PAPER

Influence of water activity and nutrients on growth and production of squalenstatin S1 by a *Phoma* sp.

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D. ALDRED, N. MAGAN AND B.S. LANE. 1999. This study investigated the effects of temperature, nutrient status and water activity (a_w) on the production of squalenstatin S1 by a *Phoma* sp. The fungus was grown on malt extract (MEA), wheat extract (WEA), oat extract (OEA) and oil seed rape extract (OSREA) agars at 15, 20 and 25 °C and 0.998, 0.995, 0.990, 0.980 and 0.960 a_w levels. The growth rate and secondary metabolite formation were followed over a total of 30 d. The maximum growth rate was observed at 25 °C and 0.998–0.990 a_w for all media types, which was significantly reduced ($P = 0.05$) for most media at 0.96 a_w . The growth rate was greatest for WEA and OEA but the growth form was an effuse exploitative type compared with the dense assimilative type on the richer MEA. The lipid-based OSREA appeared to be a poor growth substrate for this fungus. In contrast to the growth rate data, squalenstatin S1 production was maximal for all media types at slightly reduced a_w in the range 0.990–0.980. There was greater production of the secondary metabolite under significant water stress (0.960 a_w) compared with that with freely available water (0.998 a_w). Maximum production was observed in WEA. Production began earlier in WEA and OEA compared with MEA. Squalenstatin S1 production was not significantly affected by incubation temperature ($P = 0.05$). This study has shown that nutritionally depleted substrates may be usefully employed in the production of squalenstatin S1 and perhaps also for other secondary metabolites.

INTRODUCTION

Microbial metabolism, being energetically expensive, is highly regulated and normally subject to physiological control that responds to environmental factors (Vining 1990). Although the commercial production of secondary metabolites is still centred around large submerged liquid fermentations (SLF), some recent studies have looked at the influence of certain environmental factors in alternative fermentation systems.

A number of studies have investigated the effects of nutrient type by comparing metabolite production using a range of agricultural materials in solid substrate fermentation (SSF) systems (O'Neill *et al.* 1993; Balakrishnan and Pandey 1996). In fact, the use of heterogeneous 'natural' substrates in SSF

can, in some cases, result in the production of unique bioactivities (Penn 1994) and, in other cases, increase titres beyond those obtained in SLF (Barrios-Gonzalez and Tomasini 1996).

Some studies, using either solid substrates or agar media, have shown that the production of secondary metabolites is influenced strongly by water activity (a_w) and temperature and by their interaction. Examples include fumonisins (Marin *et al.* 1995), alternariols (Magan and Lacey 1985), aflatoxins (Niles *et al.* 1985), patulin (Roland and Beuchat 1984) and penicillic acid (Northolt *et al.* 1979). The vast majority of studies showed the highest metabolite production at the highest a_w level used (i.e. wettest conditions) and at the optimal growth temperature. However, Baxter (1997) and Etcheverry *et al.* (1994) reported optimal production at lowered a_w levels for squalenstatin and tenuazonic acid, respectively. Overall, the a_w ranges employed in these types of studies have varied enormously, often working at levels that could be expected to be severely inhibitory to growth.

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Recently, Baxter (1997) and Baxter *et al.* (1998) reported that both a_w and temperature stress significantly influenced the titres of squalostatins produced in surface cultures by a *Phoma* sp. The squalostatins are a group of fungal metabolites that have been shown to be potent inhibitors of cholesterol and ergosterol biosynthesis via competitive inhibition of squalene synthase (Dawson *et al.* 1992). They therefore offer potential as cholesterol-lowering drugs and as broad-spectrum antifungal treatments in animals and crops (Bergstrom *et al.* 1995). Baxter *et al.* (1998) only compared a rich defined medium (malt extract agar) with a solid maize grit substrate. No previous studies have examined weak extracts of starchy and oil-based seeds and compared this with complex defined media for stimulating secondary metabolite production. Recently, extracts of cereals have also been found to contain oligosaccharides which have been demonstrated to increase the production of penicillin G by strains of *Penicillium chrysogenum* (Asilonu 1999). However, the interaction with environmental parameters has not previously been examined.

The objectives of the present study were to investigate the effect of weak undefined starch- and oil-based seed extracts and interactions with a_w and temperature on temporal growth and squalestatin production by a *Phoma* sp. Comparisons were also made with a defined medium shown previously to significantly enhance metabolite production.

MATERIALS AND METHODS

Stock cultures

Stock cultures of the *Phoma* sp. (IMI 332962) were stored at ambient temperature in 1.8 ml sterile water in 2 ml cryogenic vials (Nalgene, Merck, Poole, UK). Working cultures were prepared on Malt extract agar plates (Lab-M, Toddington, UK) incubated at 25 °C for 14 d.

Media preparation

Starchy grain extracts were prepared from wheat and oats (R.D. Hawkings, Stagsden, UK). For both grain types 30 g grain were chopped in a blender (Waring) for 1 min on the 'high' setting. The resulting material was added to 400 ml reverse osmosis (RO) water and autoclaved for 1 h at 121 °C. The extract was allowed to cool and filtered through two thicknesses of muslin and made up to a volume of 500 ml with RO water. This suspension was used at 400 ml l⁻¹ in the final medium, equivalent to 24 g l⁻¹ original grain.

Oily grain extracts were prepared by sonicating (Soniprep-Fisher, Loughborough, UK) aliquots of 7.5 g (1%) crude rape seed oil (Cargill UK, Hull, UK) into 750 ml RO water to produce a stable emulsion.

Malt extract medium (ME) was prepared using 30 g l⁻¹

ME (Difco, East Molesey, UK) and 5 g l⁻¹ mycological peptone (Lab M).

Agars were prepared from these extracts (agar no. 2; Lab-M) at water activity (a_w) levels of 0.997, 0.990, 0.980 and 0.960 by the addition of appropriate weights of the non-ionic solute glycerol prior to autoclaving (Dallyn 1978). Glycerol was used in this study since NaCl (ionic solute) has previously been shown to have an inhibitory effect on squalestatin production (Baxter *et al.* 1998). The agar a_w was measured using a Humidat IC-2 instrument (Novasina, Pfaffikon, Switzerland) after calibration with standard salt tablets. The agar a_w was found to be at least within 0.005 of the required levels, and typically within 0.002.

Culture and growth conditions

All agar plates were inoculated centrally with one 4-mm diameter plug of *Phoma* sp. taken from the margin of growing cultures. Agar plates were divided into three groups and incubated at 15, 20 and 25 °C for a total duration of 30 d. For each experimental treatment (agar type, a_w and temperature) 10 replicate plates were incubated. Colony diameters were measured in two directions every 2–4 d. From these data mean colony radial growth rates (mm d⁻¹) and standard errors of the mean (\pm S.E.M.) were determined.

Secondary metabolite analysis

The methodologies previously developed for the extraction and quantification of squalestatin S1 were used (Baxter *et al.* 1998). Three replicate plates from each treatment were destructively sampled at 10, 20 and 30 d. In each case, 4-mm diameter plugs were removed from the colony surface in a pattern of equal spacing. Colonies greater than 25 mm diameter had 16 plugs removed; colonies of 15–25 mm diameter had seven to eight plugs removed and colonies of less than 15 mm diameter were not sampled.

Plugs were placed in 2-ml Eppendorf tubes (Sartorius, Epsom, UK) and stored where necessary at –20 °C. Metabolite extraction was carried out using an extraction solvent comprising 80% v/v acetonitrile (Aldrich, Gillingham, UK) and 0.03% v/v H₂SO₄ (BDH, Poole, UK) in water (Aldrich). One ml was added for 16 plugs, 0.5 ml for eight plugs and 0.438 ml for seven plugs. The samples were incubated at 25 °C for 1 h with shaking every 15 min. The liquid was decanted and filtered through a 0.45- μ m nylon syringe filter (Phenomenex, Cheshire, UK). Filtered supernatant fluids were transferred to 2-ml high-pressure liquid chromatography (HPLC) vials (Anachem, Luton, UK).

Squalestatin S1 was quantified using a Gilson (Villiers le Bel, France) 715 HPLC system. Aliquots of 10 μ l were injected automatically using a Gilson 231 XL sampling injector. The column used was 5- μ m C6 spherisorb

(150 × 4.6 mm; Phenomenex) with a guard column of the same material. The mobile phase was 55% v/v acetonitrile and 0.015% v/v H₂SO₄ in water. The running conditions were 1 ml min⁻¹ flow rate, total run time 20 min and u.v. detection at 210 nm. Extracts were analysed against calibration curves of S1 over the range 5–50 µg ml⁻¹. Mean yields of squalstatin S1 were calculated from the HPLC results, expressed as µg g⁻¹ wet weight agar (± s.e.m.). Analytical losses were accounted for by determining extraction efficiency. Standards were kindly supplied by Glaxo Wellcome Research and Development (Stevenage, UK).

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA; Microsoft Excel version 5 tool package). Two factor analysis was employed to investigate the effects of two variables, and their interactions, in appropriate experiments. Least significant difference (LSD) analysis was then used to identify significant differences between individual treatments in relevant experiments.

RESULTS

Effects of substrate, a_w and temperature on growth

The effect of the interaction between a_w and temperature on the linear growth rate of the *Phoma* sp. on malt extract agar (MEA) and wheat extract agar (WEA) is shown in Fig. 1. This shows a marked effect of temperature on linear growth rate when compared with the effect of a_w over the range investigated. The data for 15, 20 and 25 °C are separated into three distinct groups in each case with statistically significant differences ($P = 0.05$) between linear growth rates at each temperature for a given a_w level. The effect of a_w on linear growth rate was also statistically significant, but it had less influence than temperature, as demonstrated by the ANOVA data (Table 1). The growth rate was significantly reduced at 0.96 a_w for all media and temperatures, except for MEA at 25 °C ($P = 0.05$).

The influence of medium type and a_w on mean growth rate at 25 °C is shown in Fig. 2. For grain- and seed-derived media, growth rates were comparable for WEA and oat extract agar (OEA) and significantly lower for oil seed rape extract agar (OSREA), which appeared to be a very poor growth substrate for this organism. Maximum growth rates for all medium types were recorded at 25 °C.

Linear growth rates on MEA were significantly lower than on starchy grain-derived media, but different growth forms were observed growing on these two media types. On the nutritionally rich MEA, a dense assimilative type growth form was evident, whilst on the more nutritionally-depleted grain extract media an effuse explorative growth form was

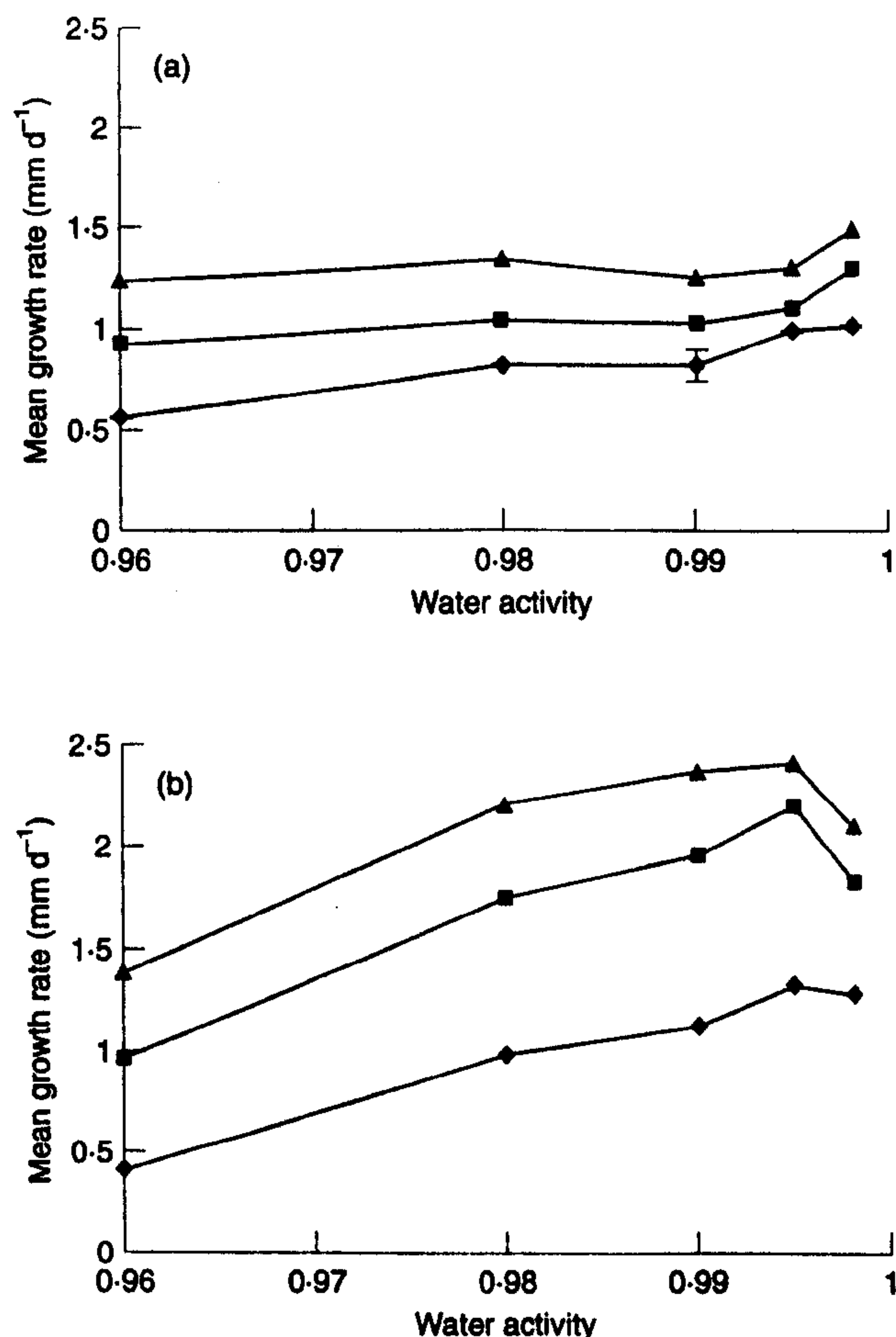


Fig. 1 Influence of temperature and water activity (a_w) on mean growth rate of *Phoma* sp. (mean of three replicates) growing on (a) glycerol modified malt extract agar and (b) wheat extract agar. Standard error bars are shown where larger than symbol size. ♦, 15 °C; ■, 20 °C; ▲, 25 °C.

noted, characterized by rapid linear expansion as defined by Rayner (1994). This may represent a physiological adaptation effected by the organism in response to the nutritional status of the environment (Cooke and Whipps 1993). Although the linear growth rate was greatest for the grain-derived media, biomass production was probably greatest on MEA.

Effects of substrate, a_w and temperature on temporal squalstatin production

Figure 3 shows the effects of the interaction between a_w and time on squalstatin S1 production for MEA and WEA, respectively, both at 25 °C. The highest yields of S1 were recorded at 30 d incubation irrespective of a_w level. This result was reflected in all experiments carried out for each medium type and temperature. It is clear from comparison

Table 1 Statistical analysis of growth data for *Phoma* sp. growing on malt extract agar (MEA), oat extract agar (OEA), oil seed rape extract agar (OSREA) and wheat extract agar (WEA) ($P = 0.05$)

Experiment	Factor	Fcrit	F	LSD
MEA	Temp.	3.2	457.4	0.050
	a_w	2.6	92.6	
	$a_w \times \text{temp.}$	2.2	7.8	
OEA	Temp.	3.2	5300.9	0.024
	a_w	2.6	3114.5	
	$a_w \times \text{temp.}$	2.2	90.2	
OSREA	Temp.	3.1	126.0	0.132
	a_w	2.5	58.9	
	$a_w \times \text{temp.}$	2.0	5.8	
WEA	Temp.	3.2	6137.8	0.031
	a_w	2.6	2093.2	
	$a_w \times \text{temp.}$	2.2	46.9	
All media, 25 °C	Medium type	2.8	495.8	0.075
	a_w	2.5	190.9	
	Medium type $\times a_w$	1.9	23.6	

LSD, Least significant difference.

of these results that S1 production on MEA, apart from being markedly lower, was reduced at days 9 and 20 in comparison to WEA. This may represent a catabolite repression effect

operating for the nutritionally rich MEA, which is less likely to appear in the nutritionally-depleted grain extract medium, or may reflect much lower levels of biomass capable of metabolite production on MEA. The broad differences in nutritional status between the media types were also apparent in the colony growth forms obtained.

Squalestatin S1 production was maximal in the a_w range 0.99–0.98 for WEA and OEA, 0.98–0.96 for MEA and 0.995 for OSREA. A similar trend was noted in all experiments carried out, with overall maximum production of S1 at sub-optimal a_w levels for growth (0.99–0.98 a_w). Statistical tests carried out on the day 30 WEA data revealed a significant effect of a_w on squalestatin S1 production ($P = 0.05$; Table 2). The LSD analysis confirmed significantly enhanced yields of squalestatin S1 at 0.99 and 0.98 a_w , with yields at 0.98 a_w being significantly higher than with any other treatment. The lowest yields were observed at 0.998 a_w (when water was freely available) at all temperatures. Although ANOVA revealed a significant effect of temperature on squalestatin S1 production overall, no clear-cut pattern could be discerned. For example, there was no significant difference between squalestatin levels at 0.998 a_w for the three incubation temperatures. At 0.995 a_w , significantly less metabolite was produced at 25 °C when compared with 15 and 20 °C. At 0.98 a_w significantly more metabolite was produced at 25 °C when compared with 15 and 20 °C ($P = 0.05$).

The yields of squalestatin are compared directly for all media types in Fig. 4 (25 °C). The enhanced levels at 0.99 and 0.98 a_w on WEA are particularly clear, together with the

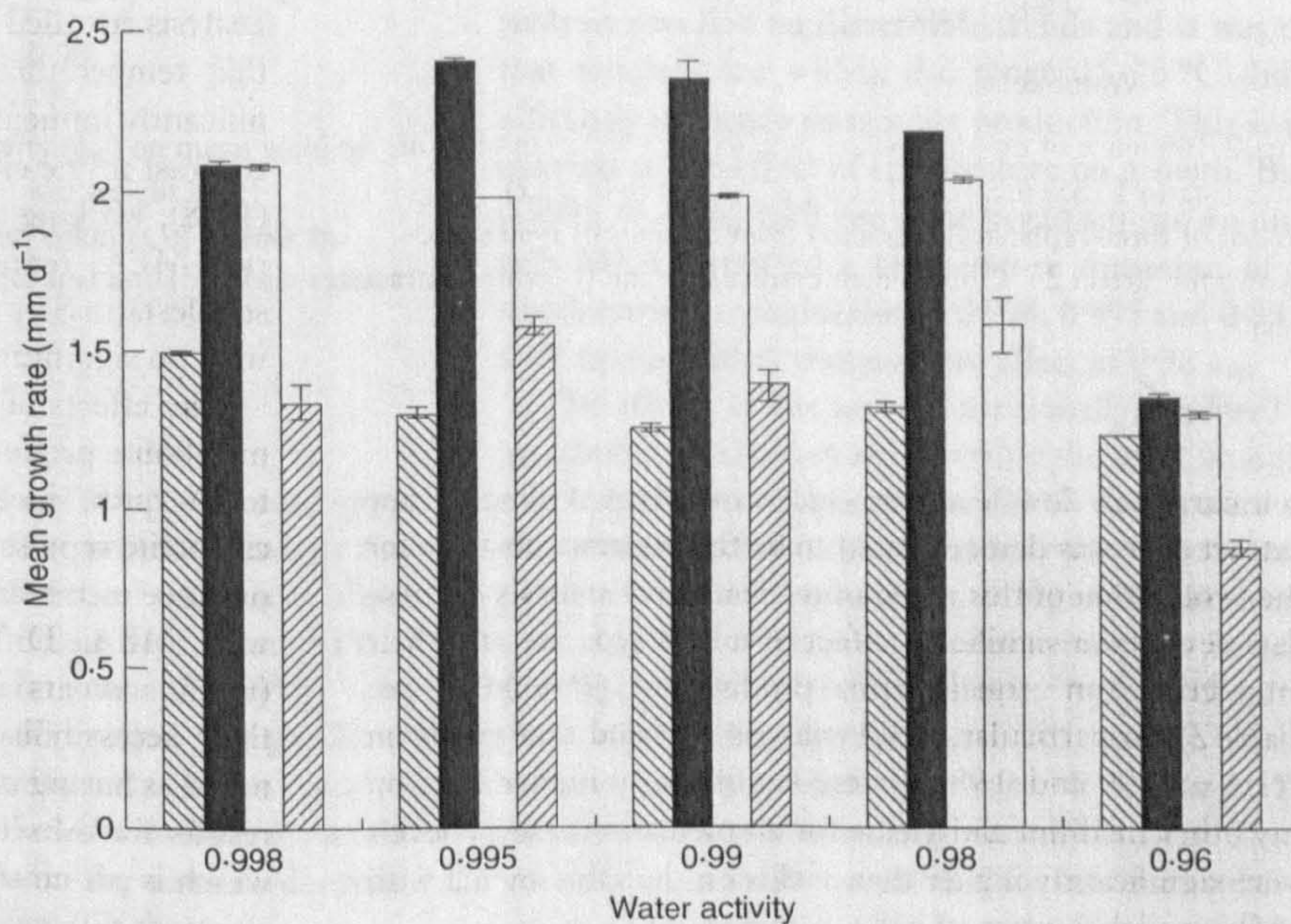


Fig. 2 Influence of medium type and water activity (a_w) on mean growth rate (mm d^{-1}) (mean of three replicates). The *Phoma* sp. was grown on malt extract agar; wheat extract agar; oat extract agar and oil seed rape extract agar for up to 30 d at 25 °C. Bars are standard errors of mean

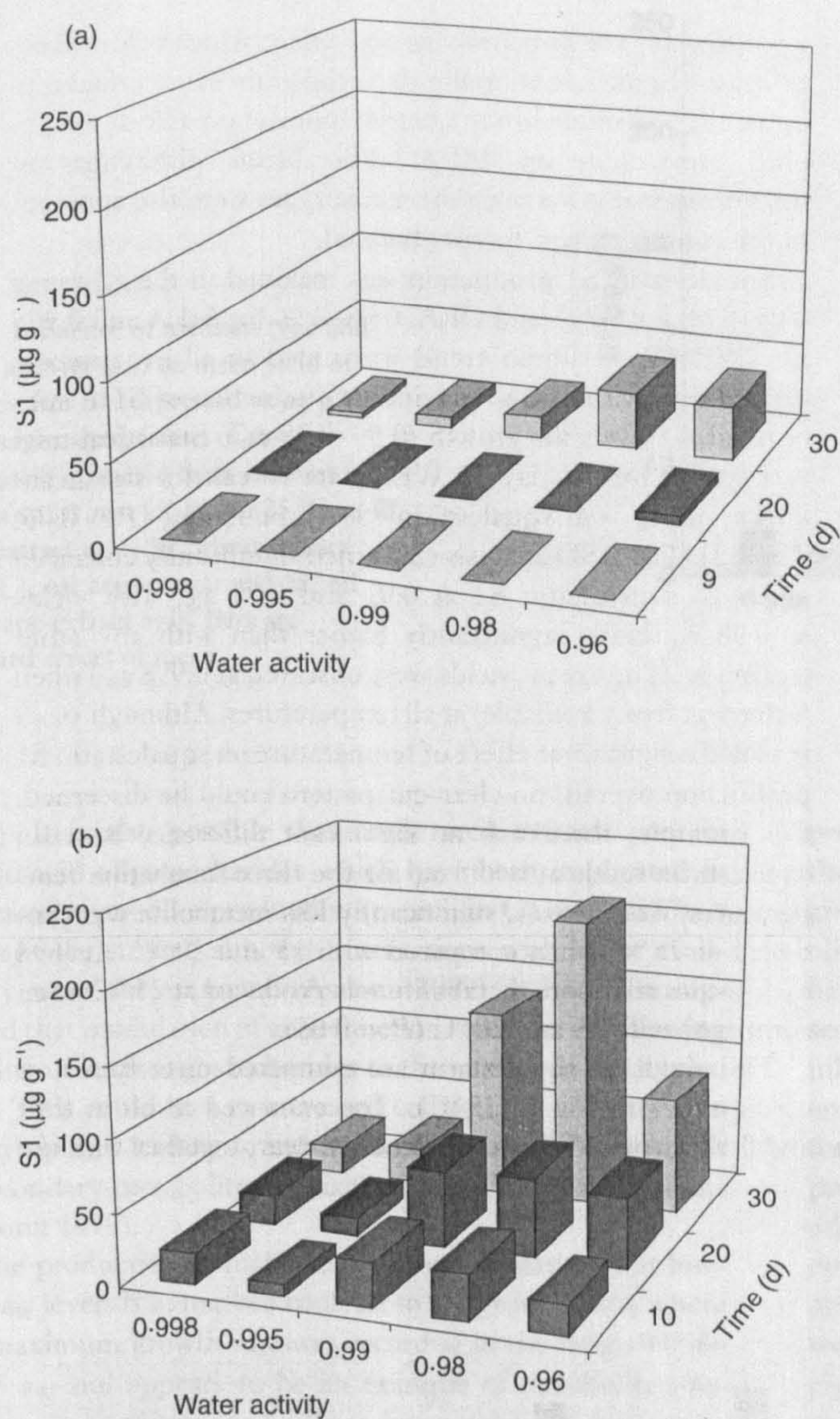


Fig. 3 Influence of time and water activity (a_w) on mean yield of squalenolone 1 (S1) (expressed in $\mu\text{g g}^{-1}$ wet weight agar) (mean of three replicates) extracted from colonies of *Phoma* sp. grown for 30 d at 25 °C on (a) malt extract agar and (b) wheat extract agar

comparatively low levels produced on MEA. Oil seed rape extract agar was demonstrated to be the poorest substrate for the production of this metabolite. Statistical analysis of these data revealed a significant effect of media type, a_w and their interaction on squalenolone production ($P = 0.05$; see Table 2). In particular, LSD analysis revealed that yields on WEA at 0.99 and 0.98 a_w were significantly higher than on any other medium and yields for all media at these a_w levels were significantly higher than yields on the same media with freely available water (0.998 a_w , $P = 0.05$).

Table 2 Statistical analysis of metabolite production data on wheat extract agar (WEA) (day 30) and all media (day 30, 25 °C) ($P = 0.05$)

Experiment	Factor	Fcrit	F	LSD
WEA, day 30	Temp.	3.3	39.3	
	a_w	2.7	143.1	
	$a_w \times \text{temp.}$	2.3	18.3	16.1
All media, day 30	Medium	2.8	80.4	
	a_w	2.6	60.7	
	Medium $\times a_w$	2.0	18.1	16.7

LSD, Least significant difference.

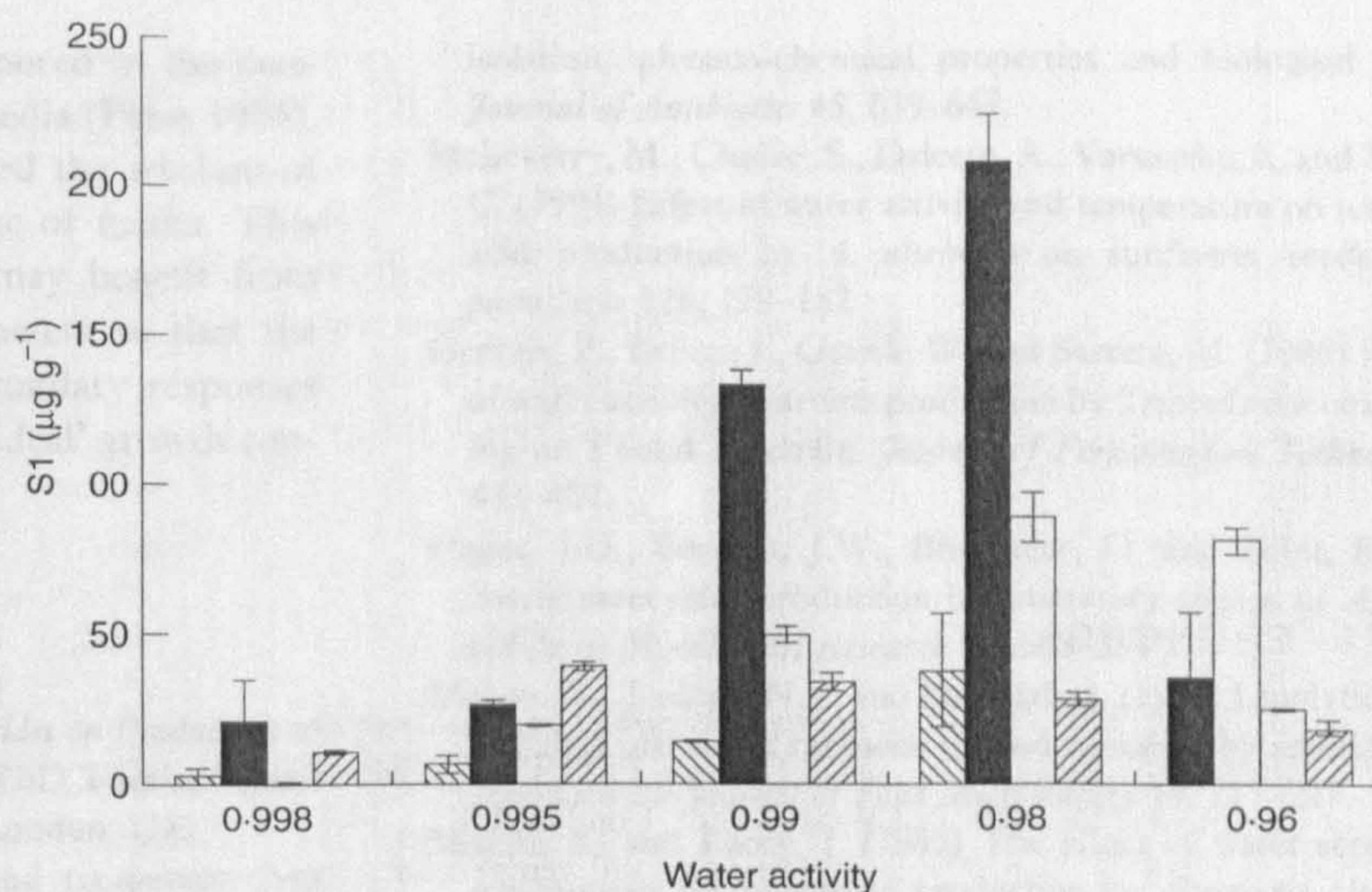
DISCUSSION

In this study, growth of the *Phoma* sp. was shown to respond strongly to temperature variation in the range 15–25 °C in comparison to the response to a_w in the range 0.998–0.96, although growth was significantly reduced at 0.96, except for MEA at 25 °C. The maximum growth rate was recorded at 25 °C, which has previously been reported as the optimum temperature for this organism (Baxter 1997). The consistently higher growth rates at this temperature at all a_w levels reflect the findings of Ayerst (1969) that the greatest 'tolerance' to lowered a_w occurs at the optimum growth temperature.

Most notably, this study has indicated that squalenolone S1 production responded to two environmental stress effects: the imposition of lower water availability (in terms of lowered a_w) and nutrition (by using nutritionally-depleted grain extract media). Although ANOVA revealed a significant effect of temperature on squalenolone S1 production overall, LSD analysis revealed no discernible trends and it was concluded that temperature within the range 15–25 °C did not significantly influence metabolite production. This is in marked contrast to the effect of temperature on growth. Baxter *et al.* (1998), working with the same organism grown on modified rich MEA, reported a temperature optimum of 20 °C for squalenolone S1 production at 0.998, 0.995 and 0.99 a_w levels, with no significant temperature effect at 0.98 a_w .

The effects of the use of nutritionally-depleted media on metabolite production are probably the most straightforward to interpret, since these are least likely to impose a significant catabolite repression effect and may therefore be expected to produce metabolite earlier and at higher levels than observed with MEA. Differences between the two starchy grains (wheat and oats) probably reflect the balance of nutrients and their accessibility during extraction; oats in particular are noted as having a tough outer 'husk' (Hajjer *et al.* 1989). The results for oil seed rape may reflect the high lipid content, which is presumably unsuitable for this particular organism, although it is rapidly utilized by spoilage fungi (Magan *et al.*

Fig. 4 Influence of medium type and water activity (a_w) on mean yield of squalenstatin S1 (expressed as $\mu\text{g g}^{-1}$ wet weight agar) (mean of three replicates) extracted from colonies of *Phoma* sp. grown for 30 d at 25 °C on ■, malt extract agar; ■, wheat extract agar; □, oat extract agar and ▨, oil seed rape extract agar. Bars are standard errors of mean



1993). It is also possible that crude seed extracts contain uronic acid oligosaccharides which have been implicated in increased metabolite production (penicillin G) by *Penicillium chrysogenum* strains, although interactions with water availability were not examined (Asilonu 1999). It has been suggested that modulation of gene function could occur following binding of such oligosaccharides to the surface of the hyphal cells. This could lead to increased cell activity on entry into the cytoplasm leading to increased biomass and biosynthesis of secondary metabolite, especially in liquid culture systems (Asilonu 1999).

The production of higher titres of squalenstatin S1 at lowered a_w levels is in marked contrast to the growth data, where the maximum growth rate was recorded in the range 0.998–0.995 a_w and appears to be an example of metabolite production responding specifically to water stress. It is also possible that the glycerol used to modify low a_w treatments had a direct stimulatory effect on metabolite production; Northolt and Bullerman (1982) found that glycerol had a stimulatory effect on the toxic secondary metabolite aflatoxin B1 (which, like S1, is a polyketide). Further, Connors *et al.* (1995) linked increased glycerol utilization with increased zaragozic acid production in submerged fermentations of *Leptodontidium elatius*. However, in the present study, the same pattern of results was obtained for all media types, including MEA where the influence of glycerol as a carbon source would be expected to be less marked, since it is unlikely to be preferentially utilized in the presence of simple sugars. In addition, Baxter (1997), working with the *Phoma* sp., reported a similar pattern of results when growing the organism on maize grits, over a range of a_w levels without the use of glycerol.

The effect of water availability stress on squalenstatin S1

production is particularly interesting. A key point here is that the manipulation of a_w in a range only slightly inhibitory to growth has resulted in a significant enhancement of metabolite production. In many previous studies, large a_w changes have been imposed so that growth and, not surprisingly, secondary metabolite production were both strongly inhibited. However, there are some studies that have reported enhanced production at a_w levels lower than those optimal for growth. For example, Gervais *et al.* (1988) studied the production of 2-heptanone (a volatile metabolite) by *Trichoderma viride* on a yeast starch agar medium and reported optimal production of the metabolite at 0.97–0.96 a_w , with optimal growth at 0.995 a_w . Similarly, Baxter *et al.* (1998), working with the *Phoma* sp. used in this study grown on glycerol-amended MEA, reported optimum a_w for growth and metabolite production at 0.995 and 0.995–0.980, respectively.

Lowering of a_w can be seen as a stress effect with direct relevance to the natural environment, in terms of the onset of drought conditions. Drought represents a major difficulty for soil-borne organisms such as the fungus used in this study, so it is perhaps not surprising to find a regulation mechanism which appears to respond to this stimulus.

Both regulatory mechanisms, i.e. catabolite repression responding to nutrient depletion and the water availability effect noted here, presumably heighten the competitive advantage of the producer in the face of deteriorating environmental conditions, assuming that the metabolite(s) so formed have a relevant extrinsic biological function. This, in turn, has practical implications for the production of fungal extracts for screening programmes. At present, little emphasis is placed on the optimization of environmental conditions in terms of precise a_w control or the imposition of nutritional

stress. Both of these factors have been ignored in the conventional use of established commercial media (Penn 1994). Tribe (1987) has also previously questioned the wisdom of the use of the mycologists' standard range of media. This study has suggested that some systems may benefit from the manipulation of environmental parameters so that the organisms are stimulated to effect the secondary responses that are normally tightly regulated under 'ideal' growth conditions.

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